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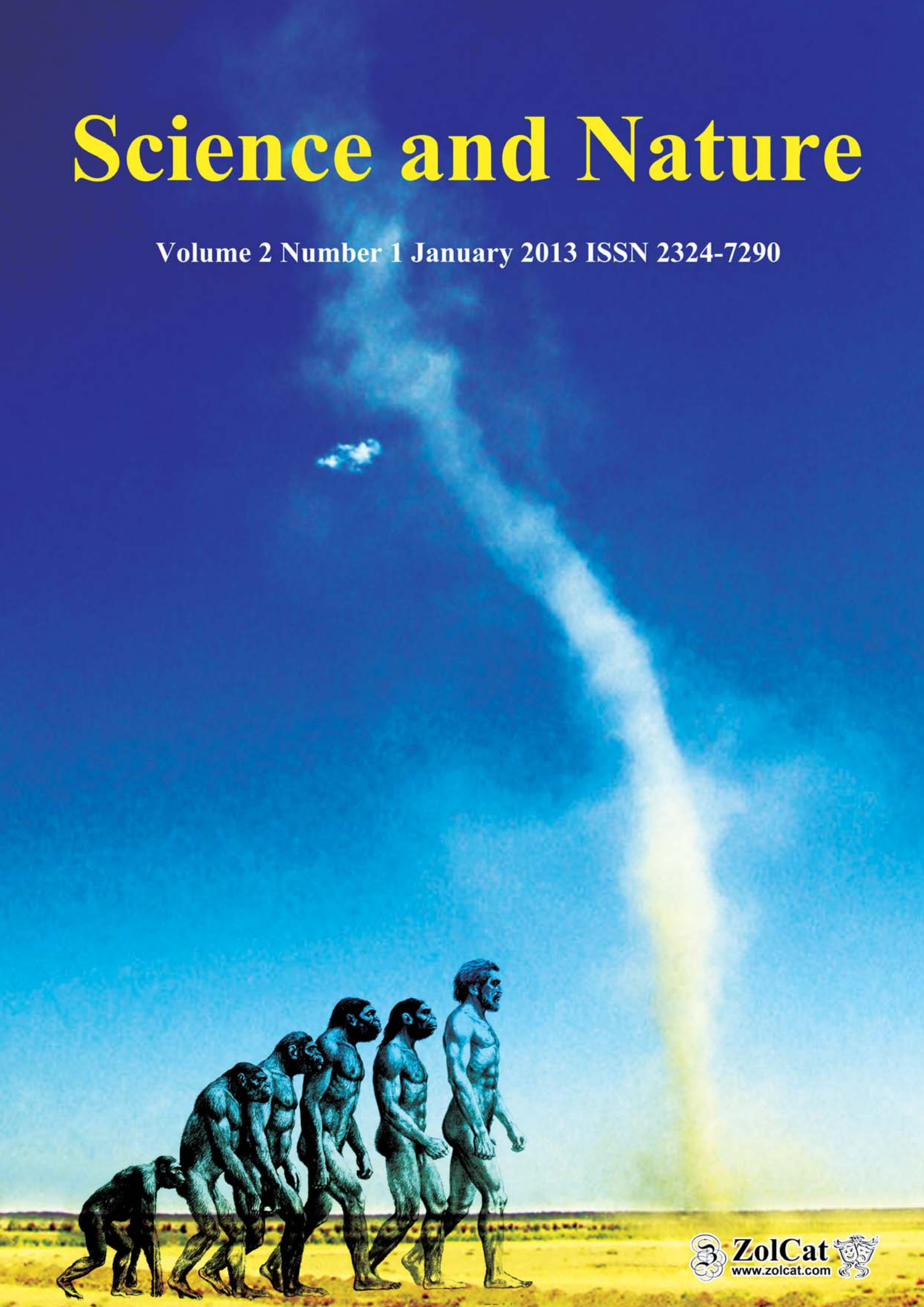

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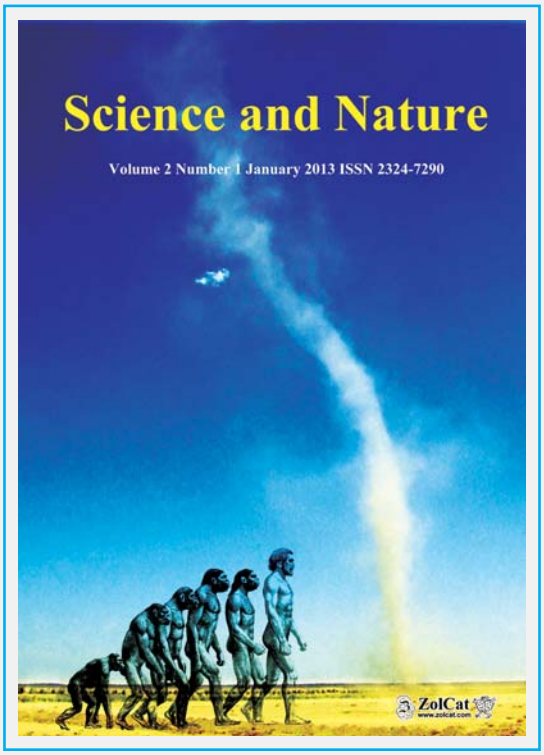

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Contents

Antibiogram and Occurence Rate of Bacteria Recovered from Pateints Attending a Fertility Clinic in Abeokuta, Nigeria

Ogiogwa I O [1], Motayo B O [1,*], Nwanze J C [2], Onoh C C [2], Adeniji F O [3], Okerentugba PO [4], Okonko I O [4]

[1] Medical Microbiology Unit, Pathology Department, Federal Medical Center, Idi-Aba, Abeokuta, Nigeria.
[2] Department of Pharmacology and Therapeutics, Igbinedion University, Okada, Edo State, Nigeria
[3] Department of Preventive and Social Medicine, College of Health Sciences, University of Port Harcourt, East-West Road, P.M.B. 5323, Choba, Port Harcourt, Rivers State, Nigeria;
[4] Medical Microbiology Unit, Department of Microbiology, University of Port Harcourt, East-West Road, P.M.B. 5323, Choba, Port Harcourt, Rivers State, Nigeria;

ABSTRACT

Infertility is a growing problem in Sub-Saharan Africa with its attendant Medical and social problems. Our study is designed to examine the Microbial pattern of Urogenital specimen of patients attending a fertility clinic in Abeokuta, Ogun State, Nigeria. Three hundred and six patients attending the Federal Medical Center Abeokuta, fertility clinic were recruited for the study, comprising 108 males and 198 females. Samples collected were semen samples from male patients and Endocervical swab samples from female patients, pregnant female patients were excluded from the study. All samples were processed following standard Microbiological protocols and antibiotic susceptibility was done by disc diffusion following the Kirby-Bauer technique. Semen samples were assessed for Morphology and sperm concentration following standard protocols. An overall isolation rate of (17.8%) was obtained for all samples processed. A total of 306 subjects were recruited consisting of 108(35.3%) male subjects and 198(64.7%) female subjects, with an isolation rate of 48(56.5%) for bacteria and 37(43.5%) for fungi (Candida albicans). Male subjects gave an isolation rate of 15(17.6%) and females 33(68.8%) for bacteria and 37(100.0%) for Candida albicans. It showed that Candida albicans (43.5%) was the only fungal isolates recovered in this study. The isolation rate of various bacteria species showed that Escherichia coli 22(45.8%) was the most predominant, followed by Klebsiella pneumoniae 12(25.0%), Staphylococcus aureus 7(14.6%), and Pseudomonas aeruginosa 5(10.4%). Proteus mirabilis 1(2.1%) and Enterococcus feacalis 1(2.1%) was least prevalent. Isolation rate of isolates by pus cell size was 68(80.0%) for normal pus cell size and 17(20.0%) for large pus cell size. Enterococcus feacalis was not isolated from sperm with normal pus cell size. Proteus mirabilis and Pseudomonas aeruginosa was not isolated from sperm with large pus cell size. Azoospermia constituted 46.3% of male subjects tested, Oligozoospermia recorded 13.9% and 39.8% of subjects had normal sperm count. Bacteria isolations were highest in Azoospermic subjects with a rate of 59.3% and lowest in Oligozoospermic subjects with 6.7%. Antibiotic susceptibility showed a high activity for Ofloxacin 74.7% and Ciprofloxacin 72.7%, Tetracycline also displayed a high level of activity 72.3%, there was high level of resistance to Cefuroxime, and others showed average susceptibility. Our study shows a high bacteria isolation rate in patients attending fertility clinic at Abeokuta and a broad diversity of organisms in urogenital specimen, therefore better attention needs to be paid to detection and treatment of all forms of urogenital infections in couples attending fertility clinics in our environment.

Key Words

Urogenital, Infertility, Bacteria, Antibiotic susceptibility, Abeokuta.

Correspondence to:

Motayo B O
Medical Microbiology unit,
Pathology Department
Federal Medical center Idi-Aba,
Abeokuta, Nigeria.
E-mail: babatundemotayo@yahoo.com

1 Introduction

Infertility can be defined as the biological inability of a man or a woman to contribute to conception. Infertility may also refer to the state of a woman who is unable to carry a pregnancy to full term (**Makar *et al.*, 2002**). Normally women experience a natural period of fertility before and during their ovulation period before returning to a natural state of infertility for the rest of their menstrual cycle (**Makar *et al.*, 2002**). Cause of infertility can be determined in about 90% of cases, but despite extensive investigations about 10% of couples never know why they cannot conceive. Between 10-30% of infertility cases have multiple causative factors, male and female infertility each account for about 30-40% of cases. In male, sperm deficit (quality and quantity) are usually responsible. Female infertility factors are more complex (**Makar *et al.*, 2002**).

Evidence has shown that urogenital infections in male and female if left untreated can lead to infertility. A good example is Clamydia trachomatis infections which are often asymptomatic in females care major causes of pelvic inflammatory disease (PID), tubal occlusion (Salpingitis), endometritis leading to infertility (**Hansfeild, 1998**). In male subjects previous studies have show a close relationship between prostititis, epidemitis, and sexually transmitted microorganisms and infertility (**Onemu *et al.*, 2010**). Also previous infection or

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existing infection of the male genital tract and assesory organs has been documented to increase the risk of infertility (***Deimer et al.*, 2003**). Clinical and experimental research has associated the recovery if bacteria isolates in semen to detioloration of spermatiogenisis and spermatozoa function which can ultimately lead to infertility (**Keck, 1998**). In sub-saharan Africa majority of cases male infertility have been traced to a previous genital tract infection or inflammation (**Onemu *et al.*, 2010**).

In Nigeria, studies have shown a relationship between positive bacteria semen cultures with poor semen quality (**Onemu and Ibeh, 2001; Emokpae *et al.*, 2005**). There is also evidence to show that increased rate of sexually transmitted infection in both males and females may contribute to the rise in infertility cases in Nigeria (**Alli *et al.*, 2011**). With the above mentioned facts we sought to investigate the occurrence rate of bacteria in urogenital samples from sexually active men and women with the objective of determining the level of isolation in relationship to infertility in Abeokuta metropolis.

2 Meterials and Methods

2.1 Study Population

A total of three hundred and six subjects, comprising 108 male and 198 female were recruited for the study all of them patients who attended the fertility clinic at Federal Medical Center Abeokuta Ogun State. Samples were collected from female patients were collected at the family planning clinic while male subjects produced semen sample from home, clinical assessment and examination were conducted at the fertility clinic by the attending physician. Pregnant women were excluded in the study.

2.2. Sample collection and Processing

Male subjects were given written or oral instruction concerning the collection and transport of semen samples. Samples were collected at the comfort of the patient's homes or a private room and transported to the laboratory within one hour. The sample was collected by masturbation and ejaculated into a clean wide mouth sterile container. High vaginal swab and Endocevical swab were collected from female patients at the family planning clinic of Fed Medical center Abeokuta and sent to the Microbiology laboratory immediately. Semen was analyzed microscopically for morphology and motility following the protocol WHO manual for the examination of semen (**WHO, 1999**). Parameters such as Appearance, Liquefaction, Viscosity and pH were recorded. Semen samples were examined under x400 magnification after the sample has been placed on a clean slide. Motility was assed by scoring either "a" Rapid progressive motility, "b" Slow progressive motility, "c" Non progressive motility and "d" Immotility. A minimum of 4 fields were viewed by a first and a second reader. Sperm concentration was determined by the WHO protocol (**WHO, 1999**) Sperm morphology was determined by making 2 smears and stained by papanicoulaou following the technique of **Meschede *et al.*** (**1993**). All samples including female endocervical swab samples were cultured on Mac Conkay agar, modified New York City agar and Chocolate agar. A wet preparation Microscopy was done for all swab samples (**Chessbrough, 1991**).

2.3. Identification of Isolates and Antibiotic susceptibility testing

Discrete colonies of isolates were picked and put into sterile peptone water, from which various biochemical tests were done to identify the organisms according to standard bacteriological practice (**Cheesbrough, 1991**). Antibiotic susceptibility testing was done using the Kirby Bauer technique for disk diffusion (**Cheesbrough, 1991**).

3 Results Analysis

Table 1 shows the frequency of occurrence of Isolates. It showed that *Candida albicans* (43.5%) was the only fungal isolates recovered in this study. The isolation rate of various bacteria species showed that *Escherichia coli* 22 (45.8%) was the most predominant, followed by *Klebsiella pneumoniae* 12 (25.0%), *Staphylococcus aureus* 7 (14.6%), and *Pseudomonas aeruginosa* 5 (10.4%). *Proteus mirabilis* 1 (2.1%) and *Enterococcus feacalis* 1 (2.1%) was least prevalent (Table 1).

Table 1. Frequency of occurrence of Isolates

Isolates	No. (%)
Candida albicans	37 (43.5)
Bacteria	48 (56.5)
Total	**85 (100.0)**

Bacteria Isolates	N=48
Escherichia coli	22 (45.8)
Enterococcus feacalis	1 (2.1)
Klebsiella pneumonia	12 (25.0)
Proteus mirabilis	1 (2.1)
Pseudomonas aeruginosa	5 (10.4)
Staphylococcus aureus	7 (14.6)
Total	**48 (56.5)**

Table 2 shows distribution of isolates in relation to sex. A total of 306 subjects were recruited consisting of 108 (35.3%) male subjects and 198 (64.7%) female subjects, with an isolation rate of 48 (56.5%) for bacteria and 37 (43.5%) for fungi (*Candida albicans*). Male subjects gave an isolation rate of 15 (17.6%) and females 33(68.8%) for bacteria and 37 (100.0%) for *Candida albicans* (Table 2).

Table 3 shows the distribution of isolates by pus cell size. Isolation rate of isolates by pus cell size was 68 (80.0%) for normal pus cell size and 17 (20.0%) for large pus cell size (Table 3). *Enterococcus feacalis* was not isolated from specimens with normal pus cell size. *Proteus mirabilis* and *Pseudomonas aeruginosa* was not isolated from specimens with large pus cell size (Table 3).

Table 4 shows the frequency of occurrence of bacteria isolates in relation to sperm cell concentration. It showed that 75.0% of the total number of bacterial isolates was recovered from those with Azoospermia. Isolation rate for Oligozoospermia (<19 x 10^6 cells/ml) was 1(2.1%) and for normal sperm count (>20 x 10^6 cell/ml) was 7 (14.6%) as shown in Table 4.

Table 5 shows the antibiotic

susceptibility pattern of the various isolates to commonly prescribed antibiotics. Values ranged from 74.7% susceptibility to Ofloxacin, 72.7% to Ciprofloxacin, 74.5% to Cotrimoxazole, 46.8% to Gentamycin and as low as 36.6% to Cefuroxime.

4 Discussion

Infertility is an increasing medical condition among married couples and also of concern among unmarried individuals in Nigeria. In Abeokuta there is also a steady increase in the attendance rate of patients with presumed infertility (Unpublished data). Lack of sufficient data in our environment concerning the relationship between cuture positive urogenital specimen and infertility viz a viz the relationship between the two prompted the conception of this study. A total of 306 subjects were studied comprising 34.5% male and 64.7% female, a total isolation rate of 85 (27.9%) with 48 (15.7%) bacteria and 37 (12.1%) fungi. This finding is similar to that of a study done in south eastern Nigeria (**Agbolahon *et al.*, 2007**).

In our study, *Candida albicans* was only isolated in female patients, this is contradictory to a study done in Benin city Nigeria which studied only male subjects and had an isolation rate of 5 (6.3%) in males (**Onemu *et al.*, 2011**). The high rate of Candia albicans isolated has been reported in our environment by several workers (**Isibor *et al.*, 2011; Nwadioha *et al.*, 2010**). The high rate of recovery of this organism has been attributed to the anatomy of the female genital tract as well as the very close proximity of the female genital tract and the anus which harbors candida.

Bacteria species distribution among subjects ranged from 22 (45.85%) for *Escherichia coli*, 12 (25%) for *Klebsiela pneumonia*, 5 (10.4%) for *Pseudomonas aureginosa* with the least prevalent organism *Proteus mirabilis* 1 (2%), *Staphylococcus aureus* gave 7 (14.5%). The bacteria distribution recorded in our study is in agreement with most studies of similar nature for instance a study by **Alli *et al.*** at Ibadan recorded a rate of

Table 2. Distribution of Isolates by sex

Isolates	No. (%)	Male (%)	Female (%)
Candida albicans	37 (43.5)	0 (0.0)	37 (100.0)
Bacteria	48 (56.5)	15 (31.3)	33 (68.7)
Total	**85 (100.0)**	**15 (17.6)**	**70 (82.4)**
Bacteria Isolates	**N=48**		
Escherichia coli	22 (45.8)	8 (36.4)	14 (63.6)
Enterococcus feacalis	1 (2.1)	0 (0.0)	1 (100.0)
Klebsiella pneumonia	12 (25.0)	3 (25.0)	9 (75.0)
Proteus mirabilis	1 (2.1)	0 (0.0)	1 (100.0)
Pseudomonas aeruginosa	5 (10.4)	0 (0.0)	5 (100.0)
Staphylococcus aureus	7 (14.6)	4 (57.1)	3 (42.9)
Total	**48 (56.5)**	**15 (31.3)**	**33 (68.7)**

Table 3. Distribution of Isolates by pus cell size

Isolates	No. (%)	Normal pus cell (%)	Large pus cell (%)
Candida albicans	37 (43.5)	31 (83.8)	6 (16.2)
Bacteria	48 (56.5)	37 (77.1)	11 (22.9)
Total	**85 (100.0)**	**68 (80.0)**	**17 (20.0)**
Bacteria Isolates	**N=48**		
Escherichia coli	22 (45.8)	17 (77.3)	5 (22.7)
Enterococcus feacalis	1 (2.1)	0 (0.0)	1 (100.0)
Klebsiella pneumonia	12 (25.0)	11 (91.7)	1 (8.3)
Proteus mirabilis	1 (2.1)	1 (100.0)	0 (0.0)
Pseudomonas aeruginosa	5 (10.4)	5 (100.0)	0 (0.0)
Staphylococcus aureus	7 (14.6)	3 (42.9)	4 (57.1)
Total	**48 (56.5)**	**15 (31.3)**	**11 (22.9)**

Table 4. Frequency of occurrence of Bacteria isolates by Sperm cell concentration

Bacteria Isolates	Sperm Concentration		
	Azoospermia N (%)	Oligozoospermia N (%)	Normalization N (%)
Escherichia coli	14 (24.1)	0 (0.0)	4 (11.6)
Enterococcus feacalis	1 (1.7)	0 (0.0)	0 (0.0)
Klebsiella pneumonia	10 (17.2)	0 (0.0)	2 (5.7)
Proteus mirabilis	1 (1.7)	0 (0.0)	0 (0.0)
Pseudomonas aeruginosa	5 (8.6)	0 (0.0)	0 (0.0)
Staphylococcus aureus	4 (6.8)	1 (6.7)	1 (2.7)
No growth	23 (40.7)	14 (93.3)	28 (80)

Key: Azoospermia(N)=58, Oligozoospermia(N)=15, Normalization(N)=35

Table 5. Antibiotic susceptibility pattern of various bacteria

Symbol code	Disc content (mg)	Resistance (%)	Intermediate (%)	Sensitive (%)
GEN	10	20 (42.5)	5 (10.6)	22 (46.8)
CXM	30	30 (63.8)	0 (0.0)	17 (36.2)
AMC	30	15 (31.9)	31 (31.9)	17 (36.2)
NIT	300	10 (21.3)	10 (21.3)	27 (57.4)
COT	25	30 (63.8)	5 (10.6)	25 (74.5)
NA	30	12 (25.5)	10 (21.3)	25 (53.2)
OFX	30	0 (0.0)	10 (21.3)	37 (74.7)
TET	30	13 (27.6)	0 (0.0)	34 (72.3)
CXC	5	15 (31.9)	10 (21.3)	22 (46.8)
AMX	25	18 (38.3)	6 (12.8)	24 (51.1)
CIP	5	0 (0.0)	10 (21.3)	37 (72.7)

25% isolation rate for Escherichia coli and *Klebseila pnuemoniae* (**Alli *et al.*, 2011**) and another study done at Lagos university teaching hospital recorded a rate of 12.1% for Esherichia coli (**Anorlu *et al.*, 2004**). The abundance of Esherichia coli and Klebseilla species can also be attributed to the close proximity of the female genital tract to the anus as these organisms are commensails of the gastrointestinal tract of humans collonising it mostly as normal flora unless in rare cases of immunosuppression where they become pathogenic.

Our study reveals that Azoospermic subjects constituted 53.7% of male population, Oligozoosspemia recorded 13.8% and normal subjects had 32.5%, this distribution is similar to a study done at Benin City which recoded a rate of 38.8% for Azoospermic and Oligozoospermic patients (Onemu et al 2010). This shows that following the strict criteria set by WHO (**WHO, 1999**) about 68% of our male study population are likely to have an unfavorable outcome with regards to achieving conception with a female partner, this follows suit to a similar study done in Lagos a low number of potential sperm donors in young adult males(**Akinrinola *et al.*, 2003**). Distribution of bacteria isolates according to sperm cell concentration revealed that, Azoospermic subjects recorded the highest isolation rate of 59.3% followed by Normal count and Oligozoospermic subjects having the lowest bacteria isolation rate. This distribution further agrees with other reports that relates recurrent bacteria infection to high risk of male infertility occurrence (**Onemu *et al.*, 2010**). The distribution of isolated organisms in relationship to pus cell size was also investigated details of result is shown in table 2, results were consistent with normal microscopic diagnostic creiteria in an infected sample (**Cheesbruogh, 1991**), with the highest isolation rate seen in large sized pus cell samples. Antibiotic susceptibility pattern is shown in table 4 reveals that the highest level of resistance was demonstrated by Cefuroxime with 63.8% while Ciprofloxacin and Ofloxacin recorded 0% resistance, sensitivity was highest in the Qunolones , with Ciprofloxacin having 72.7% and Ofloxacin 74.7%, Tetracyclin also displayed a high level of sensitivity to isolates tested with 72.3% sensitive. This shows the high activity of the Quinolones to pathogenic bacteria with a very low level of resistance development in this subpopulation of patients despite reports of abuse of this class of antibiotics. It is however worrysome to discover the high level of resistance recorded to a very potent 2nd generation Cephalosporin (Ceturoxime) in our study setting this further confirms the existence and increasing incidence of extended spectrum beta lactamase (ESBL) bacteria in Abeokuta as revealed by earlier studies (**Motayo *et al.*, 2011**).

5 Conclusion

Our study thereby reveals the presence of diverse pathogenic bacteria in samples of patients reporting for Infertility problems, thereby highlighting the importance of detection and prompt treatment of all forms of Gynaecological and Urogenital infections in couples attempting to achieving conception and looking forward to bringing fort a new life in Abeokuta metropolis.

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Diagnostic Role of Serum Glypican-3 as a Tumor Marker for Hepatocellular Carcinoma

Soha Z El-Shenawy[1], Maha M El Sabawi[2], Nashwa Sheble[2], Mona Abd El-Raof[3], Maha M Allam[4], Samar K Fath Allah[5]

Departments of [1]Clinical Biochemistry, [2]Hepatology, [3]Public Health and [4]Clinical Pathology, National Liver Institute, Minoufiya University, Egypt
[5]Department of Clinical Pathology, Faculty of Medicine, Minoufiya University ,Egypt

ABSTRACT

Hepatocellular carcinoma (HCC) is a major health problem. It has been increasing in Egypt with a doubling in the incidence rate in the past 10 years. It represents the most common primary malignant tumor of the liver and is one of the major causes of death among patients with cirrhosis. Current diagnosis of HCC relies on clinical information, liver imaging and measurement of serum alpha-fetoprotein (AFP). The reported sensitivity and specificity of AFP are not sufficient for early diagnosis, and so additional marker is needed. The development of effective marker for the diagnosis of HCC could have an impact on HCC-related cancer mortality and significant public health implications worldwide. In the adult, Glypican-3 (GPC3) can only be detected in a limited number of tissues, including the lung, ovaries, mammary epithelium, and mesothelium. It is expressed in fetal livers but not in adult livers. The soluble form of GPC3 was identified in the serum of patients with hepatocellular carcinomas, and can be used as a serological test for the diagnosis of hepatocellular carcinoma. It was reported that the frequency of GPC3 expression in AFP-negative HCC patients is as high as 90%, suggesting that it can be used in diagnostic of HCC. **The aim of the current study** was to detect the value of serum GPC3 in HCC Egyptian patients as a more specific, sensitive and accurate biomarker by comparing it with an established biomarker as AFP. **Subjects and methods:** The patients were selected from the Department of Hepatology, National Liver Institute, Minoufiya University. There were three groups (HCC group, Liver cirrhosis group and control group). The serum estimation of AFP and GPC-3 were done to all subjects. **Results:** When analysis of variance was done between the three groups, a highly statistical significant difference was found between these groups regarding the mean serum levels of both AFP and GPC-3 where the highest increase of both markers were found in the HCC group. Results of the ROC curves analysis showed that the optimal cut-off of GPC-3 to differentiate between cirrhotic patients from healthy subjects is 0.5 ng/ml with 90% sensitivity & 80% specificity and 19 ng/ml with sensitivity and the specificity 63.5% and 70% respectively to differentiate HCC patients from liver cirrhotic patients. **Conclusion:** GPC-3 could be a sensitive, specific and accurate serum marker for early diagnosis of HCC. Further studies in larger groups of patients are needed to confirm this finding.

Key Words

Hepatocellular carcinoma, HCC, Glypican-3, GPC-3, AFP.

Correspondence to:

Soha Z El-Shenawy
Department of Clinical Biochemistry,
National Liver Institute,
Menoufiya University,
Shebin El-Kom, Egypt.
E-mail: sohazaki69@yahoo.com

1 Introduction

Hepatocellular carcinoma (HCC) is a major health problem, with more than 500.000 cases diagnosed annually [1]. The burden of hepatocellular carcinoma (HCC) has been increasing in Egypt with a doubling in the incidence rate in the past 10 years [2]. Estimates of the burden of cancer caused by these factors provide an opportunity for prevention. Previously, there was strong evidence that hepatitis B virus (HBV) was the major cause of HCC in Egypt, but more recently hepatitis C virus (HCV) has become the predominant factor associated with the more recent epidemic of HCC. It has been well documented that Egypt has one of the highest prevalence rates of HCV infection in the world [3]. Other factors such as cigarette smoking, occupational exposure to chemicals such as pesticides, and endemic infections in the community, such as schistosomiasis, may have additional roles in the etiology or progression of the disease [4]. Early recognition of the onset of HCC would help to select more effective therapies for patients leading to a better prognosis and life span. Current diagnosis of HCC relies on clinical information, liver imaging and measurement of serum alpha-fetoprotein (AFP) [5].

Serum alpha-fetoprotein (AFP) was first described as a marker for HCC by **Abelev *et al.*, in the 1960** and used as a serum marker for HCC in humans

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for many years [6]. The first quantitative serum assays for AFP were established by **Ruoshlati and Seppala** [7]. It has a sensitivity of 39%–65%, a specificity of 76%–94%, and a positive predictive value of 9%–50% [8]. HCC patients with a high AFP concentration (≥ 400 IU/mL) tend to have greater tumor size, bilobar involvement, massive or diffuse types, portal vein thrombosis and a lower median survival rate [9]. Though the measurement of AFP serves as an important tool in screening of HCC, some reports have indicated that it has limited utility of differentiating HCC from benign hepatic disorders for: its high false-positive and false-negative rates, elevated levels in patients with acute exacerbation of viral hepatitis and that tumors other than HCC may also have markedly increased AFP levels like testicular tumors [9]. AFP with its reported sensitivity and specificity are not sufficient for early diagnosis as AFP concentrations are directly correlated with tumor size. So, the development of effective marker for the diagnosis of HCC could have an impact on HCC-related cancer mortality and significant public health implications worldwide[10]. Although ultrasonography has been widely used in clinical screening of HCC, it is highly dependent on the experience of its operator. Therefore, as AFP and ultrasonography only play a limited role in screening of HCC, some candidate biomarkers can be used in diagnosis of HCC including glypican-3 (GPC3) [11].

GPC3 belongs to a family of glycosylphosphatidylinositol-anchored, cell-surface heparan sulfate proteoglycans. Six glypicans have been identified in mammals so far (GPC1 to GPC6) [12]. Although the homology of amino acids between glypican members is moderate, all glypicans are approximately 60 to 70 kd in size and share a characteristic pattern of 14 conserved cysteine residues [13]. Intact glypicans are decorated with heparan sulfate (HS), which is located in the last 50 amino acids of the C terminus, placing the HS chains close to the cell membrane [14].

It is an oncofetal protein that is located on the X chromosome, and is highly expressed in the embryo and involved in morphogenesis and growth control during development[15]. It is reported that a loss-of -function mutation in the GPC3 gene causes Simpson-Golabi-Behmel syndrome, a rare X-linked disorder characterized by pre- and postnatal overgrowth, increased risk of embryonic tumors during early childhood, and numerous visceral and skeletal anomalies [16].

In the adult, GPC3 can only be detected in a limited number of tissues, including the lung, ovaries, mammary epithelium, and mesothelium [17]. So, down regulation of GPC3 has been observed in several human malignancies, including mesothelioma and ovarian, breast and lung cancers [18-21]. These observations indicate that GPC3 is an inhibitor of cell proliferation and a tumor suppressor in a tissue-specific manner [22].

GPC3 is expressed in fetal livers but not in adult livers [23]. There have been a number of studies showing that GPC3 expression is frequently up-regulated in HCCs at the messenger RNA and protein levels when compared with normal livers and benign hepatic lesions[24]. The soluble form of GPC3 was identified in the serum of patients with hepatocellular carcinomas, and can be used as a serological test for the diagnosis of hepatocellular carcinoma [25]. The results of immunohistochemical studies have convincingly shown that GPC3 is a novel diagnostic marker for HCC [26]. It was reported that the frequency of GPC3 expression in AFP-negative HCC patients is as high as 90%, suggesting that it can be used in diagnostic of HCC[27].

So, the aim of the current study was to detect the value of serum GPC3 in HCC Egyptian patients as a more specific, sensitive and accurate biomarker by comparing it with an established biomarker as AFP.

2 Subjects and Methods

Patients

In the current study, the patients were selected from the Department of Hepatology, National Liver Institute, Minoufiya University. There were three groups.

First group (HCC group)

It included 85 patients (67 males and 18 females). Their mean age was 55.74 ± 5.2 years. These patients were diagnosed as HCC by the presence of characteristic hepatic masses on liver CT, MRI and hepatic angiography (i.e., enlarged tumors and/or tumors with typical arterial vascularization. Tumor staging was determined according to the Cancer of liver Italian Program (CLIP) classification [28].

Second group (LC group)

It included 50 liver cirrhotic (LC) patients (39 males and 11 females). Their mean age was 54.84 ± 3.68 years with no radiological evidence of HCC.

Third group (Control group)

It included 35 apparently healthy subjects as a control group with no evidence of liver disease and/or neoplasm. They were 29 males and 6 females, with mean age 56.82 ± 6.17 years.

All the procedures included in this study were approved by the Research Ethics Committee of National Liver Institute, Minoufiya University, Egypt. Venous blood sample were withdrawn by venipuncture from all individuals included in this study and subjected to the following parameters: prothrombin time (PT) & concentration (using Behring Fibrin timer II, Germany), total & direct bilirubin (T. & D.Bil) and albumin (using the Beckman Coulter, Synchron C9 ALX, Clinical Autoanalyzar, USA), AFP (using VIDAS instrument, BioMerieux, France by the Enzyme Linked Fluorescent Assay). 1ml of serum of each subject involved in this study was frozen and stored until GPC3 assay. Serum level of GPC3 was determined by using Uscn Life Science Inc. Wuhan, Germany, by the enzyme linked Immunosorbent assay. By following the manufacturer's

protocol, the concentration of GPC3 in the samples is determined by comparing the optical density of the samples to the standard curve. The minimum detectable dose of human GPC3 in the kit is 0.036 ng/ml.

Statistical Analysis

Data were collected and entered to the computer using SPSS (Statistical Package for Social Science) program for statistical analysis. For comparing 2 groups, continuous normally distributed variables were tested for association by student's t-test. On the other hand, Mann–Whitney test is a non-parametric test for assessing whether two independent samples of observations have equally large values. For more than 2 groups, normally distributed variables were tested with ANOVA test. While, Kruskal Wallis test was done for variables that wasn't normally distributed.

The Pearson`s correlation coefficients were calculated for the normally distributed values. However, Spearman`s correlation coefficients were done for the not normally distributed values. Receiver Operating Characteristic (ROC) curves was produced for the measured parameters to investigate the sensitivity, specificity and the cut-off values of each AFP and GPC3. P value <0.05 and <0.01 were considered statistically significant.

3 Results

Table 1 showed the statistical comparison between HCC group, LC group and the control group regarding the mean serum albumin & total bilirubin, INR and degree of ascites & encephalitis. When analysis of the results of the mean serum level of each AFP and GPC3 in the three studied groups, a statistical significant difference (p<0.001) was detected between them where the maximum increase of both parameters was observed in the HCC group as in Table 2.

Table 3 showed the descriptive statistics of CLIP score items in the HCC patients where the mean CLIP score in these patients was 2.97 ± 1.68.

Figure 1 represented the ROC curve of GPC3 and AFP to differentiate HCC patients from liver cirrhotic patients. When analysis of the results of the ROC curve, this study found the cut-off point is 19 ng/ml for GPC3 and 78 IU/ml for AFP. The sensitivity and the specificity for GPC3 is 63.5% and 70% respectively and for AFP is 76.5% and 82% respectively as described in Table 4.

Figure 2 showed the ROC curve of GPC3 and AFP to differentiate liver cirrhotic patients from healthy subjects. By analysis of the data in the ROC curve as in Table 5, the cut-off to differentiate between cirrhotic patients from healthy subjects is 0.5 ng/ml for GPC3 with 90% sensitivity & 80% specificity and 8.5 IU/ml for AFP with 76% sensitivity & 80% specificity.

Table 6 showed a statistically significant positive correlation between GPC3 and each of AFP (r=0.593, p=<0.001), tumor size (r=0.277, p=<0.05) and CLIP score (r=0.505, p=<0.001). Also, a statistically significant positive correlation was found between AFP and each of Child score (r=0.302, p=<0.05), CLIP score (r=0.640, p=<0.001 and the tumor size (r=0.469, p=<0.001).

Regarding the mean serum level of GPC3 in HCC patients as in Table

Table 1. Statistical comparison of different studied parameters in the three studied groups

	Studied groups						Test of sign.	*P* value
	HCC N = 85		LC N = 50		Control N = 35			
	N	(%)	N	(%)	N	(%)		
Albumin (g/dl)								
< 2.8	44	51.8	19	38.0	0	0.0	113.0	<0.001
2.8 – 3.5	29	34.1	22	44.0	0	0.0		
> 3.5	12	14.1	9	18.0	35	100		
Bilirubin (mg/dl)								
< 2	42	49.4	32	64.0	35	100	37.1	<0.001
2 – 3	23	27.1	11	22.0	0	0.0		
> 3	20	23.5	7	14.0	0	0.0		
INR								
< 1.7	24	28.2	10	20.0	35	100	90.75	<0.001
1.71 – 2.1	41	48.2	35	70.0	0	0.0		
> 2.1	20	23.5	5	10.0	0	0.0		
Ascites								
Non	38	44.7	26	52.0	35	100	43.84	<0.001
Mild	30	35.3	17	34.0	0	0.0		
Mod. / sever	17	20.0	7	14.0	0	0.0		
Encephalitis								
Non	64	75.3	49	98.0	35	100	24.79	<0.001
Mild	17	20.0	1	2.0	0	0.0		
Mod. / sever	4	4.7	0	0.0	0	0.0		

P-value is highly significant at <0.001

Table 2. Statistical comparison of different studied parameters in the three studied groups

	Studied groups			Test of sign.	*P* value
	HCC N = 85 Mean ± SD	LC N = 50 Mean ± SD	Control N = 35 Mean ± SD		
Child score	9.10 ± 2.87	8.24 ± 2.30	5.0 ± 0.0	52.05	<0.001
AFP (IU/ml)	5529.0 ± 7008.6	80.4 ± 159.3	6.6 ± 3.04	72.10	<0.001
GPC3 (ng/ml)	1646.3 ± 3980.2	12.7 ± 10.4	1.3 ± 2.9	90.75	<0.001

P-value is highly significant at <0.001

Table 3. Descriptive statistics of CLIP score items in HCC group

	N=85	(%)
Child score		
5 – 6	19	22.4
7 – 9	27	31.8
10 – 15	39	45.9
Tumor		
Uninodular	25	29.4
Multi nodular < 50	42	49.4
Multi nodular > 50	18	21.2
AFP (ng/ml)		
< 400	50	58.8
> 400	35	41.2
Portal vein invasion		
Yes	24	28.2
No	61	71.8
CLIP score (Mean ± SD)	2.97 ± 1.68	

Table 4. ROC curve analysis of the studied parameters (GPC3 and AFP) to differentiate HCC patients from liver cirrhotic patients

	GPC3 (ng/ml)	AFP (IU/ml)
Area under the curve	0.753 (<0.001)	0.758 (<0.001)
95 % CI	0.672 – 0.834	0.677 – 0.840
Cut-off point	19.0	78.0
Sensitivity	63.5%	76.5%
Specificity	70.0%	82.0%
Positive predictive value	78.3%	87.8%
Negative predictive value	53.0%	67.2%
Accuracy of the test	65.9%	78.5%

Table 5. ROC curve analysis of GPC3 and AFP differentiate liver cirrhotic patients from control subjects

	GPC3 (ng/ml)	AFP (IU/ml)
Area under the curve	0.897 (<0.001)	0.813 (<0.001)
95 % CI	0.834 – 0.960	0.723 – 0.904
Cut-off point	0.50	8.50
Sensitivity	90.0%	76.0%
Specificity	80.0%	80.0%
Positive predictive value	81,8%	79.2%
Negative predictive value	88.9%	76.9%
Accuracy of the test	85.0%	78.0%

Table 6. Correlation analysis between each of GPC3 and AFP with different parameters in HCC patients (N=85)

	GPC3 (ng/ml)		AFP (IU/ml)	
	(r)	***P* value**	**(r)**	***P* value**
Child score	+ 0.161	>0.05	+ 0.302	<0.05
Tumor size	+ 0.277	<0.05	+ 0.469	<0.001
AFP (IU/ml)	+ 0.593	<0.001		
Portal vein invasion	+ 0.431	<0.001	+ 0.455	<0.05
CLIP score	+ 0.505	<0.001	+ 0.640	<0.001

P-value >0.05 isn't significant P-value is significant at <0.05 & <0.001 Spearman,s correlation was used for all the variables except for AFP and LIP score as Pearson,s correlation was done.

Table 7. Statistical comparison between each of GPC3 and AFP with different parameters in HCC patients (N=85)

	GPC3 (ng/ml)			AFP (IU/ml)		
	GPC3 (ng/ml) Mean ± SD	**Test of sign.**	***P* value**	**AFP (IU/ml) Mean ± SD**	**Test of sign.**	***P* value**
Child score						
5 – 6	225.94 ± 312.05	0.18	>0.05	2294.0 ± 3860.11	2.72	>0.05
7 – 9	1464.59±2702.38			5462.33±6082.92		
10 – 15	2464.17±5315.91			7151.17 ±8262.65		
Tumor						
Uni nodular	316.48 ±324.96	11.17	<0.001	3572.64±4437.38	22.36	<0.001
Multi nodular<50	935.47±2265.11			3536.23±6108.05		
Multi nodular>50	5152.11±6992.50			12895.94±7213.51		
AFP (IU/ml)						
< 400	76.92± 153.39	7.66	<0.001	71.06 ± 57.83	7.81	<0.001
> 400	3888.40±5505.43			13326.06 ±3868.23		
Portal vein invasion						
Yes	531.16 ±1320.54	3.95	<0.001	3650.29±5974.74	4.17	<0.001
No	4480.79±6452.06			10304.04±7288.42		

P-value >0.05 isn't significant P-value is significant at <0.05 & <0.001

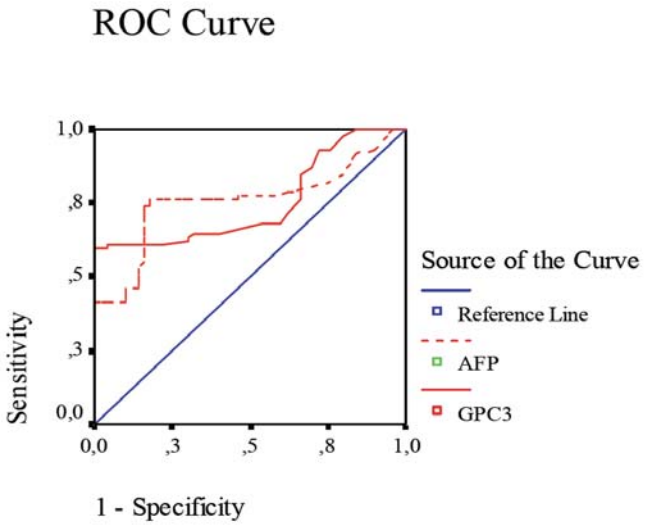

Fig.1 ROC curve of GPC3 and AFP to differentiate HCC patients from liver cirrhotic patients

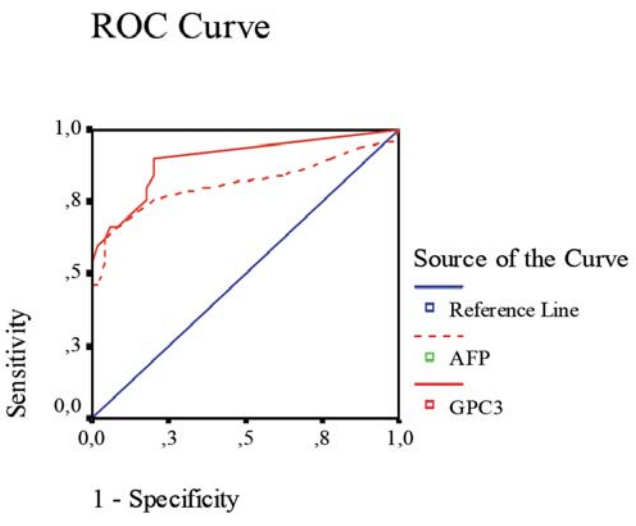

Fig.2 ROC curve of GPC3 and AFP to differentiate liver cirrhotic patients from control subjects

7, it showed a statistically significant difference (p<0.001) with tumor size and presence of portal vein invasion. Considering the mean serum level of AFP in the same patients, a statistically significant difference (p<0.001) was found with the same parameters. While no statistically significant difference was detected between each of GPC3 and AFP with Child score.

4 Discussion

Hepatocellular carcinoma (HCC) is characterized by a multi-cause, multi-stage and multi-focus process of tumor progression. Its prognosis is poor due to both its late detection and the lack of effective therapies for advanced stage disease (29-30). Up to 80% of HCCs develop against a background of cirrhosis of the liver and the surveillance of the at risk cirrhotic population could aid earlier detection of the disease and decrease the cancer related mortality rate [31]. Currently, standard surveillance includes a combination of 6 monthly abdominal ultrasound scan and serum alphafetoprotein measurement, but this strategy does not reliably detect early disease (32). The aim of this study was to evaluate the value of serum GPC(3) in HCC Egyptian patients.

Regarding the mean serum level of AFP, a highly statistically significant difference was observed between the three groups (HCC, Liver cirrhosis and control groups). A marked increase showed in the HCC group, while a slight increase occurred in the cirrhotic group. In the current study by applying the ROC curves, analysis showed the best cut-off value for AFP to differentiate HCC patients from cirrhotic patients was 78 IU/ml. This gave 76.5% in sensitivity and 82% in specificity. While the best cut-off recorded in this study to diagnose liver cirrhosis was 8.5 IU/ml. It yielded a 76% sensitivity and 80% specificity. **Soresi *et al.*** [33] showed that the best cut-off value of AFP has been reported to be 30 IU/ml (sensitivity of 65%, specificity of 89%) in Sicilian population compared with 200 IU/ml (sensitivity of 70%, specificity of 100%) in Burman population. However, **Zhou and his colleagues**, [34] reported that soma investigations have showed that the cut-off value is fluctuant in different ethnic groups and one of possible reasons for this difference is the diverse living circumstance which has a great influence on epidemiology. They also reported that AFP is more useful in detecting HCC patients with non-viral etiology. **Lau and Lai** [35] stated the specificity of AFP is very high when the levels are above 400 IU/ml in patients without testicular tumor. Also, **Goma *et al.*** [8] revealed an AFP value above 400-500 IU/ml has been considered to be diagnostic for HCC in patients with cirrhosis. Therefore, all these results indicate that serum AFP level plays a limited role in diagnosis of HCC, especially early HCC.

Regarding the mean of the serum level of GPC3, The present study showed a highly statistically significant difference was observed between the studied three groups with highest increased in the HCC group and a slight increase only occurred in the cirrhotic group. Moreover, the appropriate cut-off value for serum GPC3 that distinguishes between HCC patients from cirrhotic patients was >19 ng/ml; it yielded a 63.5% sensitivity and 70% specificity. While the best cut-off that differentiates cirrhotic patients from control subjects was >0.5 ng/ml, it gave a 90% sensitivity and 80% specificity. **Liu and his coworkers** [11] agreed with this study as they stated that GPC3 can be used as a potential biomarker for the diagnosis of early HCC and can be used in screening of HCC as they found that the serum GPC3 level was higher than 300 ng/l in 50% of early HCC patients, although their serum AFP level was below 100 μg/L in their study. They recorded that at cut-off 300 ng/L for GPC3, the sensitivity and specificity for the diagnosis of HCC was 46.7% and 93.5% respectively. **Shafizadeh *et al.*** [27] found GPC3 positive cells in 90% of patients with their serum AFP level <400 μg/L. They also found that serum GPC3 level was increased in early HCC patients with their serum AFP level <400 μg/L. So, they concluded that GPC3 is a sensitive, specific serum and tissue marker for the diagnosis of early HCC. Also, **Nakatsura *et al.*** [36] demonstrate that the expression of GPC3 (at both mRNA and protein levels) in the serum of HCC patients is significantly higher than that in serum of healthy adults (p<0.001) or patients with nonmalignant hepatopathy (p<0.01), and it can be detected in 40-53% of HCC patients and 33% of HCC patients with seronegative for AFP. **Yao *et al.*** [30] concluded that an oncofetal antigen GPC3 and GPC-3 mRNA expression in hepatocarcinogenesis is a promising molecular markers for early diagnosis of HCC, especially in poor-differentiated or small HCC.

However, **Beale and his colleagues**[37] revealed that GPC3 has no role at all in the surveillance of HCC in individuals with steatohepatitis related cirrhosis as they found both the sensitivity and specificity of GPC3 were poor in their patient set.

At the current study, a positive correlation was found between serum level of each of AFP and GPC3 with both tumor size and portal vein invasion. This comes in accordance with **Zhou *et al.*** [34] who stated that HCC patients with a high AFP (≥400 ng/ml) tend to have greater tumor size, bilobar involvement, massive or diffuse types, portal vein thrombosis, and a lower median survival rate.

In conclusion, GPC-3 could be a sensitive, specific and accurate serum marker for early diagnosis of HCC. Further studies in larger groups of patients are needed to confirm this finding.

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Isolation and Characterization of Protease Producing Marine Eubacteria

Singh K, Bose H, Richa K, Karthik L, Gaurav K, KV BhaskaraRao*

Molecular and Microbiology Research Laboratory, Environmental Biotechnology Division, School of Bio Sciences and Technology, VIT University, Vellore-632014, Tamilnadu, India

ABSTRACT

Twenty five bacterial isolates from soil samples collected from coast of Andhra Pradesh were screened for protease production, among which 8 strains showed proteolytic activity and one isolate (N2) was selected for further study. The proteolytic bacteria was identified as Bacterium VITKHRB based on morphological, biochemical tests and 16s rDNA sequencing. Enzyme was produced, purified up to 1.20 fold and its specific activity was found to be 1.83 IU/mg. Therelative molecular mass of enzyme was measured 36 kDa by SDS-PAGE. The best enzyme activity was observed at pH 8 and temperature 35°C, 6.5% NaCl concentration, xylose as carbon source and yeast extract as nitrogen source. This enzyme is expected to be a good industrial application as it was found to digest egg white and remove blood stain efficiently. This is the first report on protease production from marine eubacteria inhabiting coast of Bay of Bengal near Andhra Pradesh.

Key Words

Protease, Eubacteria, *Bacterium VITKHRB*, SDS-PAGE.

Correspondence to:

KV BhaskaraRao

E-mail: kokatibhaskar@yahoo.com

1 Introduction

Enzymes are the biocatalysts that are known for enhancing rate of metabolic reaction by lowering the activation energy in our body. A number of enzymes being important in vivo processes and have industrial importance too. Among these, protease is one of the industrially important enzymes. It is a naturally occurring enzyme present in all organisms constituting 1-5% of total protein content. It is present in all living organisms including human beings, plants, insects even micro organisms like bacteria, actinobacteria, viruses etc (**Karthik *et al.*, 2011**). Protease has been also reported in many pathogenic and infectious micro-organisms. It is responsible for proteolysis i.e., protein catabolism by hydrolysis of peptide bonds that link amino acids together in the polypeptide chain. They themselves being protein are cleaved by other and even same variety of protease molecules. They are capable of hydrolysing almost all protein as long as they are not the component of living cells. They are of great importance as they can lead to activation of a function or can be signal in a pathway. Extracellular proteases are important for the hydrolysis of external proteins and enable the cell to absorb and utilize the hydrolytic products. Simultaneously, they can be destructive as they can abolish a protein's function or digest it to its principal components. Moreover, they are simultaneously, they can be destructive as they can abolish a protein's function or digest it to its principal components. Moreover, they are also considered as one type of exo toxins. Besides this they are of great value in food, detergent, leather, pulp and paper industry (Anonymous). They constitute about two-third of total enzymes used in industries for various purposes. It is also used in bioremediation processes. Microbial proteases are very important in wide variety of biotechnological applications and account for approximately 59 % of total enzymes used. Among bacteria, the *Bacillus* sp. is specific producers of extracellular proteases (**Joshi, 2010**). The lack of pathogenicity and the ability to grow in simple culture medium can also be accounted for their applications in industry (**Daniel *et al.*, 1984**).

Marine environment has been natural habitat for organisms known for bioactive compounds viz, enzymes, antibiotics, biosurfactants, etc. It has been found to be highly variable in respect of different parameters viz salinity, temperature, pressure, density, light and even sound because of which the bioactive substances synthesized by its inhabitants are also capable to sustain or retain their activity in such a flexible or extreme conditions (**Karthik *et al.*, 2011**). These organisms may be various plants, animals, fungi and even microorganisms. In fact now-a-days microorganisms are more focussed as compared to plants and animals for enzymes of industrial importance as they have been found to be more stable than enzymes derived from plants and animals. Unusual characteristics can be seen in these enzymes due to their habitat related properties such as salt tolerance (above 1.7M) (**Marhuenda**

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et al., **2002**), hyperthermostability (80-108°C), barophilicity (60 MpA), cold adaptivity (Esterase can retain its 50% activity at freezing point of water) and pH. They also have novel chemical and sterochemical properties (**Trincone, 2011**). Enzymes derived from extremophilicarchaea have higher stability towards heat, pressure, detergents, solvents and they are often more resistant to cellulolytic attack (**Egorova *et al.*, 2005**). Marine enzymes have also been used in pollution monitoring (**Van der Oost *et al.*, 2003**). In a review by **Trincone** (**2010**) the entire marine enzyme has been highlighted. A number of extremophiles have been isolated from marine environments which can thrive on wide range of pH, salt concentration and pressure and they have enzymeswhich can act upon carbohydrates, proteins and lipids (**Antranikian *et al.*, 2005; Demirjian *et al.*, 2001; Ferrer *et al.*, 2007**). Several screening techniques and processes methodology are being adapted for isolation and detection of potent micro-organisms for production of enzymes with novel physiological properties (**Karthik *et al.*, 2010**). It has been found that marine bacteria has an unique light harvesting pigment called as Proteorhodopsin which mediates phototrophy that allows their survival even during starvation period. In present study we report the production Protease from marine eubacteria isolated from the soil samples collected from coast of Bay of Bengal near Andhra Pradesh. Earlier few groups have reported the effective production of marine eubacterial enzyme. But this is the first report of enzyme production from marine eubacteria belonging to coast of Bay of Bengal near Andhra Pradesh. The enzyme produced can be utilized effectively as an industrial application.

2 Materials and methods

Chemicals

All the mediaused for this study and dialysis membrane was purchased from Hi Media chemicals, Mumbai, India and chemicals were from Merck Specialities Private Limited, India and Sisco Research Private Limited, Mumbai, India respectively.

Sample collection

Salt pan soil samples were collected from Ongole (15°30'N, 80°03'E) which is situated at coastal regions of Andhra Pradesh in sterile polybags at a depth of about 3-4 cm with the help of a sterile spatula. The bags were transferred to the labs in sterile conditions and were stored at 4°C till isolation.

Isolation of Marine Eubacteria

Isolation was done by serial dilution method. Plating was done by spread plate method on Nutrient Agar medium supplemented with 50% distilled water and 50% marine water. The plates were incubated for 24-48 hrs at 37°C.

Screening of potent Protease producing strains

The isolates were screened for proteolytic activity by growing them on Nutrient Agar Medium supplemented with 1% Casein and Skim milk. Casein is a type of phosphoprotein which is prepared by acetic acid precipitation. Skim milk powder is a milk made protein supplement which is made by dehydrating milk after the removal of its major fat components. These two proteins are generally used for screening of protease. After incubation period plates were observed for clear zone around the colonies.

Identification of protease producing bacteria

Cultural characterization

The isolates were observed under the microscope, the colony morphology was noted down with respect to colour, shape, size, nature of colony and pigmentation.

Microscopic observation

The bacterial isolates were stained by Gram staining and observed under a high power magnifying lens in Light microscope. Endosporestaining and Capsule stainingwere performed to observe the morphology of the cells.

Biochemical characterization

The bacterial isolates were characterized biochemically by Indole test, Methyl red test, VogesProskauer test, Simmons Citrate test.

Molecular characterization

The strains were screened on the basis of above tests and the most efficient isolate was characterised based on 16S rDNA sequencing. Phylogenetic tree was constructed using the Tree view software.

Production of Enzymes

Fermentation medium for protease was prepared. Protease production media was prepared which contained, (g/L) Dextrose 10, Peptone 5, KH_2PO_4 2, $MgSO_4.7H_2O$ 2, Casein 10, at pH 8. Enzyme production was carried out by inoculating 10ml of bacterial inoculums in 500 ml production medium and the flask was kept on rotary shaker incubator at room temperature for 24 hours. After incubation, fermented broth was centrifuged at 10000 rpm for 10 minutes in a cooling centrifuge. Supernatant was collected and used for estimation of protease.

Optimization of temperature, pH, carbon source, nitrogen source and NaCl concentration on protease enzyme productivity and enzyme activity

Effect of temperature on enzyme production and enzyme activity was studied by adjusting the incubation temperature at 25, 30, 35, 40, and 45°C and production medium pH 5.5, 6.0, 6.5, 7.0, 7.5 and 8.0. Similarly, effect of carbon source, nitrogen source and NaCl concentration was studied by adjusting different carbon sources (Sucrose, Fructose, Xylose, Starch and Lactose), nitrogen sources (Beef extract, Yeast extract, Nutrient Broth, Urea and Casein) and NaCl concentrations (2.5, 3.5, 4.5, 5.5, 6.5 %) in the production medium (**Ashwini *et al.*, 2010**).

Protein Estimation

Protein estimation was done using Lowry's method (**Lowry *et al.*, 1951**).

Enzyme Assay

The original casein assay was first described by **Kunitz** (**1947**) and later

modified by **Detmar and Vogels (1971)**. 0.1 ml of enzyme was taken in test tube and 0.9 ml of 0.1 N TrisHCl (pH 8) was added to it. 1 ml of 1% Casein was added and it was incubated at 37°C for 30 minutes in a water bath. 2 ml of Trichloroacetic acid (TCA) was added to stop the reaction. The reaction mixture was centrifuged at 5000 rpm for 10 min. Supernatant was collected and absorbance was measured at 280 nm by spectrophotometer.

Partial purification

Ammonium Sulphate precipitation

The 100 ml of cell free extract was saturated with ammonium sulphate. The desired saturation of 80% was achieved. The contents were centrifuged at 5000 rpm for 20 min and pellet was collected. The supernatant was saturated to 90% again. Again the contents were centrifuged at 5000rpm for 20 minutes. Now the supernatant was discarded and pellet was collected for further analysis.

Dialysis

The precipitate was desalted by dialysis. The enzyme solution was placed in a bag of selectively permeable membrane (Dialysis membrane-150) One end of the dialysis bag was tightly tied and the precipitate recovered was taken inside the bag. The other end of the dialysis bag was tightly tied to prevent the leakage. After that, dialysis bag was suspended in a beaker containing 0.5 M Tris-HCL buffers (pH 8) for 24 hours.

SDS PAGE

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was carried out according to the method of Laemmli (1970) with slight modification using a 10% cross linked polyacrylamide gel.

Application of Protease

Digestion of natural protein

The crude enzyme (10 ml) was incubated with coagulated egg white at room temperature at different incubation time.

Removal of Blood Stain

Few drops of human blood were taken on a clean piece of cloth and blood clot was allowed to dry. Then the cloth was incubated with crude protein at room temperature for 24 hours. After incubation cloth was rinsed with water for 2 minutes and then dried. The same procedure was done with control (detergents).

3 Results

In the present study a total of 4 marine sediments were collected and processed by serial dilution and spread plate method. A total 25 different bacterial strains (Figure 1) were isolated from the marine sediments of Bay of Bengal in the coastal regions of Andhra Pradesh. Out of these 25 isolates, 8 isolates showed proteolytic activity. Among them a strain N2 was selected based on its ability to produce largest zone of hydrolysis on Casein Agar plate. According to the results of primary screening strain N2 was chosen for production.

Identification of protease producing bacteria

The bacterial isolates were characterized on the basis of colony morphology, microscopic characteristics and biochemical tests. Taxonomical identification of the bacterial isolate was performed by 16S rDNA analysis. The 16S rDNA sequence of the bacteria was blasted using online tool blast of NCBI gene bank and the phylogenetic tree was constructed with other homologous sequences (Figure 2). According to the results obtained from morphological, biochemical (Table 1) and 16S rDNA sequence characteristics, the isolate had 97% similarity with *Bacillus* sp and named as *Bacterium VITKHRB* (Acc.no: JN656215).

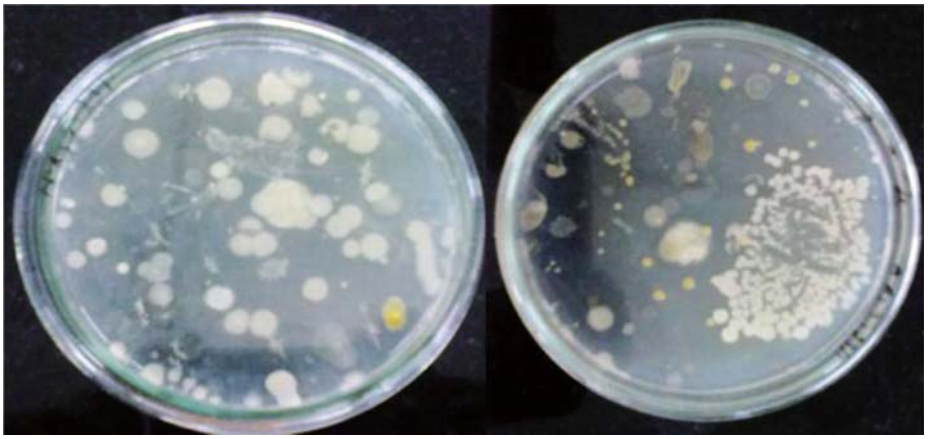

Fig.1 Isolation of eubacteria

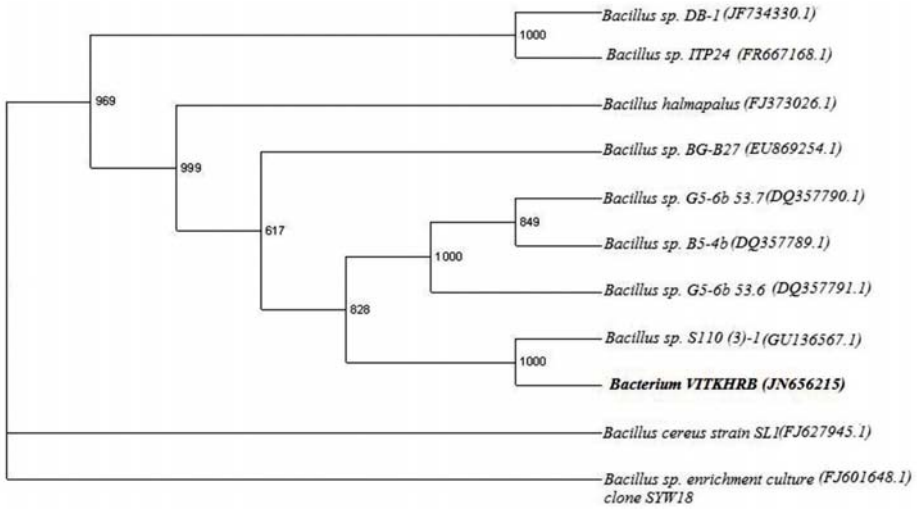

Fig.2 Phylogenetic tree of *Bacterium VITKHRB*

Effect of temperature on enzyme activity and protein content

Enzyme activity was maximum at 35°C. Enzyme production decreased as temperature was increased to 40°C and above (Figure 3).

Effect of pH on enzyme activity and protein content

Maximum enzyme activity was observed at pH 8 and as the pH was increased or decreased, there was gradual decrease in growth of the organism, protein content and enzyme activity (Figure 4). Organism did not grow at pH below 5 and above 10.

Effect of carbon sources on enzyme activity and protein content

The effect of Carbon source on protease production was characterized using five different sugars at 1 % (w/v) concentration. Maximum enzyme activity was observed in the presence of xylose as carbon source, whereas, the minimum enzyme activity and protein content was observed in the presence of fructose (Figure 5).

Effect of nitrogen source on enzyme activity and protein content

Production of protease has been studied in presence of five different organic and inorganic nitrogen sources. Among all, yeast extract showed maximum enzyme activity as compared to other organic nitrogen sources (Figure 6).

Effect of NaCl concentration on enzyme activity and protein content

At 6.5 % NaCl concentration, the enzyme activity was found maximum (Figure 7). As the NaCl concentration decreased, there was gradual decrease in enzyme production, enzyme activity and growth of organism.

Partial purification of Protease

Table 1. Morphological and biochemical characteristic of *Bacterium VITKHRB*

	Characterization of bacteria	Result
Culture characteristics	Colony Morphology on Nutrient Agar Medium	Medium, circular, off white mucoid, colonies
Microscopic characters	Gram staining	Gram positive, rods
	Endospore staining	Terminal Endospore forming
Biochemical characters	Indole	Negative
	Methyl Red	Negative
	VogesProskauer	Positive
	Citrate Utilization	Positive
	Catalase test	Positive
	Oxidase test	Positive
	Starch Hydrolysis	Positive

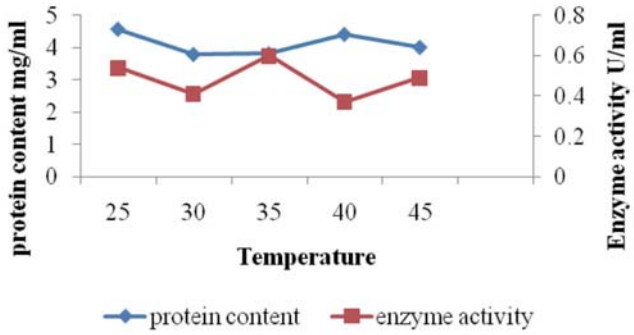

Fig.3 Effect of temperature on enzyme activity and protein content

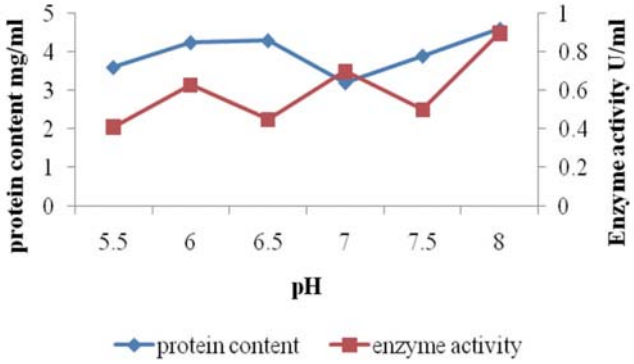

Fig.4 Effect of pH on enzyme activity and protein content

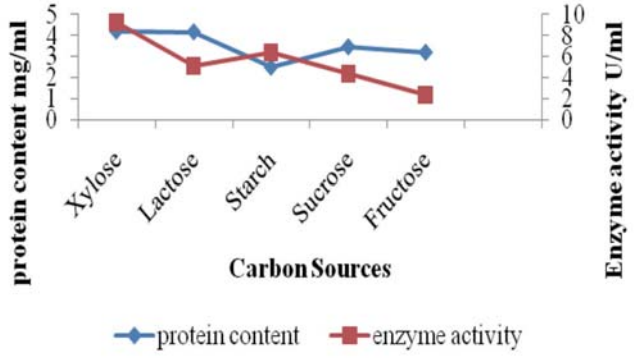

Fig.5 Effect of carbon sources on enzyme activity and protein content

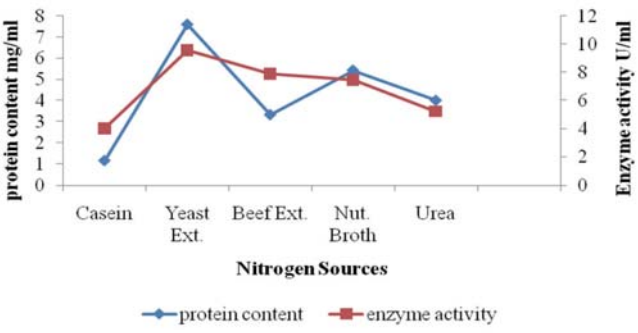

Fig.6 Effect of nitrogen source on enzyme activity and protein content

Table 2. Partial purification of protease from *Bacterium VITKHRB*

Purification steps	Total protein (mg/ml)	Enzyme Activity (mg)	Specific Activity (U/ml/mg)	Fold Purification	Yield (%)
Crude Extract	5.50	8.20	1.50	1.00	100
Ammonium precipitation	0.72	1.23	1.70	1.13	15
Dialysis	0.30	0.55	1.83	1.20	6.7

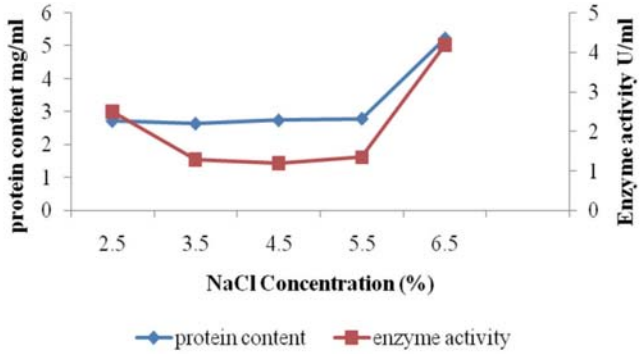

Fig.7 Effect of NaCl concentration on enzyme activity and protein content

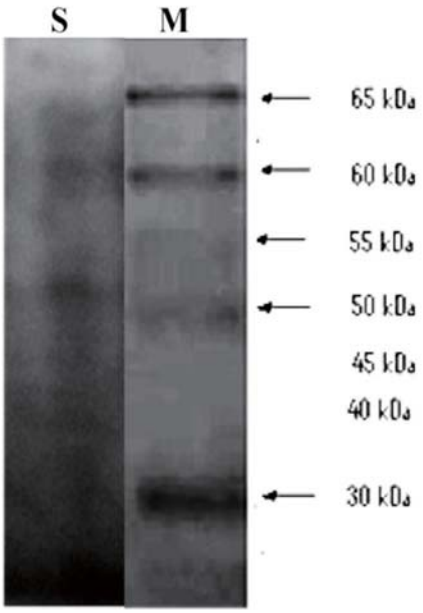

Fig.8 Polyacrylamide gel electrophoresis of partially purified sample (S – sample; M – Protein Marker).

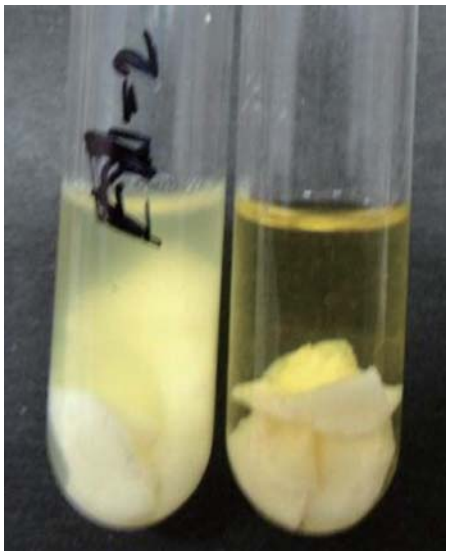

Fig.9 Degradation of egg white

Partial purification of protease enzyme was performed by Ammonium sulphate precipitation followed by dialysis. Increase in enzyme activity was observed after ammonium sulphate precipitation and dialysis. Partially purified protease exhibited specific activity of 1.83 U/ml/mg which corresponds to 1.20 purification fold and 6.7 % Yield (Table 2).

SDS-PAGE

SDS-PAGE was performed for the partially purified protease after dialysis (Figure 8). Multiple bands were observed in the gel at 36kd, 50kd and 60kd etc as the protein was not fully purified and only partially purified (Dialysis). The partially purified protein has to be subjected to further purification steps such as ion-exchange chromatography etc.

Digestion of Natural Protein

The egg white was completely digested after 24 hours of incubation at room temperature. Since the egg white contains coagulase so the protease may be of coagulase type (Figure 9).

Removal of Blood Stain

After 24 hours of incubation at room temperature partial removal of blood stain was observed on the cloth treated with the crude enzyme. Stain removal rate was found to be high as compared to control.

4 Discussion

Proteases are considered among the most important enzymes to be produced commercially and are of great significance. They have their applications in food, detergent pharmaceuticals etc. Earlier several studies reported the biological production of protease from marine microorganisms. The present study is a preliminary screening report of soil samples obtained from coastal regions of Andhra Pradesh. Number of isolates was more as compared to earlier reports (**Elela *et al.*, 2011**). This suggests that the coastal regions of Andhra Pradesh are potent source of industrially important microorganisms. Out of all the isolates the isolate with the highest zone of inhibition was selected for protease production. It was identified as *Bacillus* spand named as *Bacterium VITKHRB*. Earlier reports also show efficient protease from *Bacillus* sp. (**Das and Prasad, 2010; Joshi, 2010; Senthilraja, and Saravanakumar, 2011**) isolated from different sources. Enzyme activity and production was checked in different conditions of temperature, pH, different carbon sources, different nitrogen sources and salt concentration. In case of temperature the results obtained are in accordance with that of results reported by **El-Kastawy** in **1998**. Different optimum incubation temperatures were reported by other investigators such as 35°C (**Gerze *et al.*, 2005**), 50°C (**Ammar *et al.*, 1991; Ali, 1991**) 65°C, and 70°C (**Sookkheo *et al.*, 2000**). As far as pH is concerned the results are correlating with the other reports obtained for the optimum pH for enzymatic activity of other *Bacillus* species: pH 7.5 for *Bacillus subtilis* ITBCCB 148

(**Yandri *et al.*, 2008**), *Bacillus* sp. HS08 (**Huang *et al.*, 2006**) and *Bacillus* sp. S17110 (**Jung *et al.*, 2007**), pH 8.0 for *Bacillus cereus* KCTC 3674 (**Kim *et al.*, 2001**), Thermophilic *Bacillus* SMIA2 (**Nascimento and Martins, 2004**) and *Bacillus cereus* BG1 (**Ghorbel-Frikha *et al.*, 2005**). Results observed in case of different carbon sources agree with one report which suggested that sources of carbon affected production of enzymes by bacteria (**Juhasz *et al.*, 2003**). Starch cause low protease production. This in accordance with one report which showed low protease production in presence of starch (**Jadeja and Bhatiya, 2010**).This is in contrast to one report which showed that starch caused high level of enzyme expression in *Bacillus* species (**Mahmood *et al.*, 2000**). It has been reported that pure sugars affected protease production considerably (**Dahot, 1993**).Production of protease in presence of different nitrogen sources are contrast to the result obtained by **Yang and Lee (2001)** which showed highest enzyme activity in presence of Beef Extract.

Related studies also reported that protease production by *Bacillus stearothermophilus* F1 and *Bacillus mojavensis* was best in the presence of organic nitrogen sources (**Razzak *et al.*, 1995; Beg and Gupta, 2003**) respectively. However, some organisms responded to organic nitrogen sources and found to be better nitrogen sources both for growth and protease production (**Aleksieva *et al.*, 1981; Phadatare *et al.*, 1993**). The organism shows a good growth and adaptability at 6.5% NaCl concentration. It also maintains high enzyme activity as compared to other concentrations, which is highly preferred for commercial purposes. Maximum enzyme activity at 1.5% NaCl concentration is in accordance with the earlier reports (**Shaheen *et al.*, 2008**). The enzyme was purified upto 1.20 fold by ammonium sulfate cut and dialysis and its specific activity was increased to 1.83 U/mg/ml which is very high thus this strain can be used for commercial production of protease. The increase in protease activity by using ammonium sulfate is same as reported earlier (**McKevitt *et al.*, 1989; Sexton *et al.*, 1994**) .In literature, the alkaline proteases with molecular weight ranging from 16-36 KDa are reported from *Bacillus* sp. (**Kaur *et al.*, 1998; Adinarayana *et al.*, 2004; Jaswal and Kocher, 2007; Almas *et al.*, 2009; Joshi, 2010**). So it can be concluded that the band observed at 36kd is of protease as compared to earlier reports. Protease produced by this isolate can be efficiently used for industrial purposes for digestion or removal of proteins. It can also be used for detergent production since it is efficiently removing the blood stain. This is in accordance with the earlier reports which also show efficient digestive properties of protease (**Malathi and Chakraborty, 1991**).The protease produced is a coagulase protease since it can efficiently digest the egg white. So this enzyme can be utilized effectively as an industrial application.

5 Conclusion

Proteases are considered among the most important enzymes to be produced commercially and are of great significance. They have their applications in food, detergent pharmaceuticals etc. Earlier several studies reported the biological production of protease from marine microorganisms. The present study reports production of protease from *Bacterium VITKHRB*. The best enzyme activity was observed at pH 8 and temperature 35˚C, 6.5% NaCl concentration, xylose as carbon source and yeast extract as nitrogen source. The protease enzyme produced is also capable of purified upto 1.20 fold and its specific activity is 1.83 U/mg/ml which is very high thus this enzyme can be utilized effectively as an industrial application.

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An Assessment of Dietary Intake Associated with the Coronary Heart Disease among Adults in Yerevan, Armenia

Ezatollah Fazeli Moghadam [1,*], Artashes Tadevosyan [2], Masood Kimiagar [3], Maryam Chamari [4]

[1] Lorestan University of Medial Sciences, Khoram Abad, Iran. PhD student, Yerevan State Medical University after M. Heratsi, Public Health Department, Yerevan, Armenia
[2] MD, PhD, DSc, Yerevan State Medical University after M. Heratsi, Public Health Department, Yerevan, Armenia
[3] PhD full Professor, Shahid Beheshti University of Medial Sciences, Nutrition Science Department
[4] MSc, Tehran University of Medial Sciences, Nutrition Science Department

ABSTRACT
Epidemiologic studies have demonstrated the relationship between the dietary intake and coronary heart disease (CHD) in various countries. Extreme changes have occurred in lifestyles as well as dietary patterns in industrialized countries. Also, no study has been done to address the association between CHD and food consumption in these populations. This case-control study was conducted to assess the dietary intake in individuals with and without CHD during 2010 and 2011; we randomly selected 320 patients with CHD and 320 subjects without CHD (≥ 30 years old) from the hospitals, polyclinics and center of preventive cardiology in Yerevan. Dietary intakes with 135 food items over the previous 12 months were evaluated using a semi-quantitative food frequency questionnaire. We observed an inversely significant association between fruits, vegetables (not potatoes), whole grain, and plant food consumption and CHD. In a logistic regression, after adjusting for confounder risk factors, each 100 g increase in fruit or vegetables decreased 63% odds of CHD. The odds ratio for those with intake of sweet and dessert in the highest quartile was 2.64 (95% CI 1.65-4.21). 85% of cases and 81.3% of controls, consumed fish and seafood less than 200 g/wk (P>0.05), also, low intake of whole grain (below 100 g/d) was most common both in cases (95.9%) and controls (93.4%). This pioneering study indicates which fruit, vegetable intakes, whole grain and plant food independently associated with the CHD risk in the population under investigation.

Key Words
Assessment, Dietary, Intake, Coronary, Heart, Disease, Adult.

Correspondence to:

Ezatollah Fazeli
Moghadam, Yerevan State Medical University after M. Heratsi, Public Health Department, Yerevan, Armenia
Tel: +37494175448, +989166663374
fax: +986613206940
E-mail: ztfazeli@yahoo.com

1 Introduction

Coronary heart disease (CHD) is one of the most common causes of morbidity and mortality in different communities (**Hadaegh *et al.*, 2009**). Many risk factors for cardiovascular disease (CVD), including high blood cholesterol, hypertension, obesity, and diabetes are substantially influenced by dietary factors (**Liu *et al.*, 2000**). Dietary intake and food habits are recognized to play key roles in the prevention and treatment of CHD (**Lancaster *et al.*, 2006**). During the past decade, numerous key epidemiologic studies related to dietary intake and CVD have been published that suggested a strong association between CHD and dietary factors (**Jakobsen *et al.*, 2009; Holmberg *et al.*, 2009; Xu *et al.*, 2006**). The recent and drastic changes that have occurred in industrialized countries have led to unhealthy dietary patterns (**Lairon *et al.*, 2005**). Findings showed differences in dietary intake and risk of CHD and related health conditions among ethnic subgroups of Blacks living in the United States (**Lancaster *et al.*, 2006**). Furthermore factors such as genetic predisposition as well as changing lifestyle including physical inactivity may also increase the coronary risk profile (**Ghosh *et al.*, 2003**). As far as Armenia is concerned, there have been no studies investigating food pattern variables in explaining risk factors for CHD. This pioneering study was carried out to evaluate dietary intake in individuals with and without CHD in this country.

2 Materials and Methods

Participants

This Observational Case-Control study was conducted during the period of March 2010 to February 2011 in the Yerevan State Medical University (YSMU) hospitals, polyclinics and in the Center of preventive cardiology. Patients aged ≥ 30 years as the case group (n=320) with established CHD were identified by cardiologist. The controls (n=320) consisted of individuals aged ≥ 30 years without CHD who attended for check-up in hospitals and polyclinics in Yerevan.

All participants were given a written informed consent prior to their participation in the study. The inclusion criteria were as follows: subjects, who attended YSMU hospitals and polyclinics and in the Center of preventive cardiology for check-up and age > 30 years. Patients were excluded if they had any previous history of MI, admission for angiography, and previous history of any kind of heart surgery or angioplasty for CHD. Pregnant women and patient with history of systemic diseases according to the medical records were also excluded. The study protocol was approved by the Ethics Committee of the Yerevan State Medical University.

Data collection

In this study we assessed family history of diabetes, heart diseases, hypertension, socioeconomic status, lifestyle factors (including smoking habits, physical activity, alcohol drinking),

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and dietary intake for each subject. Next, anthropometric measures (height, weight, and hip and waist circumferences) were obtained. Waist and hip measures were assessed by using a soft tape measure, with waist measures taken at the midpoint between the costal margin and ileac crest and hip measures taken at the widest circumference. Finally systolic and diastolic blood pressure was measured twice with a standard mercury sphygmomanometer after the participants sat for 15 min; the mean of the two measurements was considered to be the participant's blood pressure at the time of health check-up.

Assessment of dietary intake

Information on the usual intakes of foods and dishes over the previous year was estimated using a semi-quantitative food frequency questionnaire (FFQ). Nutritionists and Public health specialists assisted to determine constructing a list of foods which ultimately consisted of approximately 135 foods and beverage items with a standard serving size that commonly consumed by Armenians. Before the FFQ was implemented in the study, it was adapted to Armenian conditions and was field-tested on 50 individuals.

Food items were classified into eleven categories: fruits (17 items), dried fruit (6 items), vegetables (23 items), meat, fish, poultry, egg and legumes (17 items), Dairy Products and Fats (18 items), miscellaneous (9 items), beverages (13 items), bread, cereal and potato (12 items), nuts (9 items), dishes and soups (8 items), and fast food (3 items). Subjects were asked to select their frequency of consumption and portion sizes of each food item in the past year on a daily (egg, bread), weekly (egg, rice or meat, vegetable, fruit), or monthly (egg, fish) basis by using household measures. If the subject did not consume that item, the "never" box was marked. For each subject, mean intake according to grams per day of each indicated categories were calculated.

Statistical analysis

A special database was developed to store and analyze the obtained data. The data collected through the questionnaire were entered into the database. Dietary variables are presented as means and standard deviations for normally distributed parameters. Comparison of numeric data was made using unpaired t-tests for normally distributed variables and the chi-square test for category parameters. To assess associations of CHD with food intake we calculated the lowest and highest quartiles of food groups. In logistic regression analysis, the odd ratio was computed according to the quartile value. All statistical analyses were done using the SPSS (Statistical Package for Social Sciences, Version 15). All p-values reported were based on two-sided tests, and the statistical significance was defined as $p < 0.05$ for all tests.

3 Results

Table 1 shows CHD risk factors of cases and controls. 50.6% of the patients with CHD were males and 49.4% were females. Also 44.1% of the controls were males and 55.9% were females. Cases had significantly higher prevalence of hypertension, MetS, current smoking, current alcohol consuming, and family history of CHD, while no statistically significant differences were found for obesity.

Table 2 shows the average consumption and standard error of each food group (g/d) for cases and Controls. In our study, the cases had significantly higher intakes of refined grain, sweets and dessert but lower intakes of fruits, vegetables (not potatoes), green leafy vegetables, fish, and seafood, nuts and plant food compared to the control group. No significant difference was observed in legumes, egg, whole grain and animal food intakes between individuals with and without CHD.

Distribution of case and control groups based on consumed food items is presented in Table 3. Among the food groups, the strongest associations were observed for fruits and vegetables. Percent of individuals who consumed fruits and vegetables less than 200 g/d was significantly higher in patient with CHD (case, 85% vs. control, 43.8%; P<0.000; case, 88.1% vs. control, 59.7%; P<0.0001 respectively). Reversely the percentage of cases who consumed processed meat ≥60 g/wk and egg ≥120 g/wk was significantly higher than that of the control group (cases, 33.1% vs. controls, 23.8%; P<0.05; cases, 27.8% vs. controls, 19.7%; P<0.05 respectively), while no statistically significant differences were found in distribution of cases and controls regarding the whole grain < 100 g/d, and fish < 200g/wk. P>0.05).

Participations defined by the CHD risk factors were calculated according to the mean of fruit and sweet & dessert intake (Table 4). In each category, the

Table 1. Characteristics of Patients with CHD and Controls

Variable	CHD group		Controlsteria		*P* value
	Males N (%)	Females N (%)	Males N (%)	Females N (%)	
Number	162(50.6)	158(49.4)	141(44.1)	179(55.9)	0.11
Current Smoking	108(66.7)	20(12.7)	66(46.8)	1(0.6)	0.000
Current Alcohol Consumption	124(70.9%)	51(32.3)	77(54.6)	29(16.2)	0.000
Hypertension	120(74.1)	130(82.3)	82(58.2)	124(69.3)	0.000
MetS	126(77.8)	129(81.6)	87(61.7)	135(75.4)	0.004
Family History of CHD	21(13)	24(15.2)	3(2.1)	7(3.9)	0.000
Obesity (BMI≥30)	65(40.1)	72(45.6)	57(40.4)	76(42.5)	0.40

Comparisons were based on the chi-squared test. P-value is for group differences after controlling for gender.

Table 2. Characteristics of Patients with CHD and Controls

Food consumption	Group		*P* value
	Cases (n=320) Mean (SE)	Controls (n=320) Mean (SE)	
Fruits	129.1 (3.96)	223.4 (6.77)	P<0.0001
Vegetables (not potatoes)	140.1 (3.21)	215.8 (6.78)	P<0.0001
Green Leafy Vegetable	4.9 (0.23)	11.4 (1.05)	P<0.0001
Fish and Seafood	16.0(0.92)	19.7 (1.57)	P<0.05
Legumes	12.4 (0.80)	14.7 (0.99)	P>0.05
Egg	17.3 (1.08)	16.7 (1.12)	P>0.05
Whole Grain	25.3 (1.99)	30.0 (2.28)	P>0.05
Refined Grain*	181.3 (6.23)	157.1(4.36)	P<0.05
Nuts	14.2 (1.28)	22.8 (1.91)	P<0.0001
Sweets and Dessert **	34.8 (2.19)	24.7 (1.48)	P<0.0001
Animal Food	427.2(10.62)	441.2(10.74)	P>0.05
Plant Food	625.1(13.18)	791.3(16.64)	P<0.0001

** Refined grain included white wheat (lavash and matnakash), loaf, toast, rolls, macaroni, and rice.*
***Sweets and dessert included candy, chocolate, sugar, jam, jelly, cake, cookies, and ice cream.*

CHD group had a lower intake of fruit than controls. Also, other subgroups of cases had higher sweet and dessert consumptions except in current smokers.

Table 5 depicts the odds ratio and 95% confidence interval for CHD according to food items intake. Among the food groups included in logistic regression analysis that adjusted to calorie and gender, intake of sweet and dessert was observed to be risk factor for CHD. The odds ratio for those with intake of sweet and dessert in the highest quartile were 2.64 (95% CI 1.65-4.21), but fruit, vegetable, whole grain, and plant food (highest vs. lowest quartile) intake proved to be inversely significantly associated with CHD. More individuals in the CHD group were in the lowest quartile of them while, there were no statistically significant interactions for nuts and animal food consumption (highest vs. lowest quartile) with CHD.

Table 3. Food Consumption in Case and Control groups

Food consumption	Group		*P* value
	Cases n=320 (%)	Controls n=320 (%)	
Fruit < 200 g/d	272 (85)	140 (43.8)	P<0.0001
Vegetable <200 g/d	282 (88.1)	191 (59.7)	P<0.0001
Total Fruit & Vegetable < 400 g/d	287 (89.7)	156 (48.8)	P<0.0001
Whole Grain < 100 g/d	307 (95.9)	299 (93.4)	P>0.05
Fish < 200g/wk	272 (85)	260 (81.3)	P>0.05
Processed Meat ≥60 g/wk	106 (33.1)	76 (23.8)	P>0.05
Egg ≥120 g/wk	89 (27.8)	63 (19.7)	P>0.05

4 Discussion

To our knowledge, this is the first epidemiologic study that evaluated the association between dietary intakes with CHD in Armenia. In this case-control study, as expected, in the t-test analysis, it was revealed that mean of fruit, vegetable, fish and sea food, and nuts intake was lower in the CHD group, whereas there was no significant difference for egg, legume, whole grain and animal food consumption in both groups.

We found that the mean daily intake of fruit and vegetable differed significantly between the two groups (case, 129.1 g/d vs. control, 223.4 g/d; case, 140.1 g/d vs. control, 215.8 g/d, respectively). The inverse association of fruit and vegetable intake with the CHD risk in some prospective cohort studies has been shown (**Fung *et al.*, 2008; www.plosone.org, 2012**). One of the most important dietary recommendations in relation to potential health gains and the elimination of CHD to a large extent in the individuals aged below 70 years is "eat ≥400 g of vegetables and fruits per day" (**Kromhout *et al.*, 2002**) while in the sample under study 89.7% of cases and 48.8% of controls consumed total fruit and vegetable less than 400 g/d (Table 3). Recently, the American Heart Association has recommended a diet that includes at least 4.5 servings of fruits and vegetables daily (**www.heart.org, 2012**). In the present study, in logistic regression analysis, the odds ratio of individual with total fruit and vegetable intake in the highest quartile compared to the lowest were 0.06 (95% CI 0.03– 0.1) and 0.1 (95% CI 0.06- 0.15), respectively, after adjusting for sex and calorie (Table 5). Also, these were independently associated with CHD after they were adjusted for other risk factors including calorie, gender, smoking and exercising. For each 100 g increase in fruit or vegetable consumption, there was a 63% reduction in the odds ratio of CHD (data not shown). A metaanalysis of 9 cohort studies reported a weaker association, i.e. a 4% lower risk of the CHD incidence for each additional portion of fruit and vegetables (**Dauchet *et al.*, 2006**). B.C.Zyriax et al, in Germany reported a 30% decline risk for every 100 g consumption of fruit and vegetable (**Zyriax *et al.*, 2005**). In a case-control study among Indian population, persons consuming a median of 3.5 serving/wk green leafy vegetable had a 67% lower relative risk than did those consuming 0.5 servings/wk. (**Rastogi *et al.*, 2004**).

A diet rich in fruits and vegetables due to a higher content of antioxidants, folate, and flavonoids has beneficial effects on markers of inflammation and oxidative stress which may inhibit the development of atherosclerosis and may result resulting in lower cardiovascular risks (**Holt *et al.*, 2009**).

In this study, we also found that the mean of nuts intake particularly sunflower nuts, was significantly higher in controls than that of the case group (controls, 22.8 g/d vs. cases, 14.2 g/d; P<0.0001) (Table 2). But based on the

Table 4. Mean and Standard Deviation (SD) of Fruit and Sweet and Dessert Intakes in Subgroup

Variables	Fruit intake (g/day)		*P*	Sweet & dessert intake (g/day)		*P*
	cases mean (SD)	controls mean (SD)		cases mean (SD)	controls mean (SD)	
Obese	141.5 (78.7)	219.2 (123.7)	P<0.0001	37 (34.2)	21.2 (21.7)	P<0.0001
Hypertension	120 (64.7)	212.6 (118.3)	P<0.0001	32 (31.2)	20.5 (20.4)	P<0.0001
MetS	129.7 (71)	223.1 (122)	P<0.0001	33.27 (30.5)	21.51 (21.61)	P<0.0001
Family History of CHD	131.9 (95)	291 (182.9)	P<0.0001	41.8 (26.1)	24.2 (17)	P<0.05
Current Smokers	120.1 (66.2)	201.2 (105.4)	P<0.0001	39.2 (52.1)	37.1 (39.7)	P>0.05
Current Alcohol Consumers	119.3 (65.4)	216.6 (132.5)	P<0.0001	31.8 (29.7)	25.1 (21)	P<0.05

Table 5. Distribution of Cases and Controls in the Highest and Lowest Quartiles, the Odds Ratio (95% Confidence Intervals) According to Food Groups Intakes

Food groups intake	Cases N (%)		Controls N (%)		Odds ratio	95% CI
	1st quartile	4th quartile	1st quartile	4th quartile		
Fruit (g/day)	116 (84.7)	21 (15.3)	44 (24)	139 (76)	0.06	0.03 – 0.10
Vegetable (g/d)	170 (78.7)	46 (21.3)	60 (26.1)	170 (73.9)	0.10	0.06 – 0.15
Sweet-Dessert(g/d)	63 (39.6)	96 (60.4)	97 (60.2)	64 (39.8)	2.64	1.65 – 4.21
Whole grain (g/d)	91 (56.2)	71 (43.8)	69 (43.7)	89 (27.8)	0.58	0.36 – 0.91
Nuts (g/d)	84 (26.3)	64 (43.2)	76 (44.2)	96 (55.8)	0.65	0.41 – 1.03
Animal Food	80 (55.2)	65 (44.8)	80 (45.7)	95 (54.3)	0.83	0.49 – 1.41
Plant Food	90 (67.7)	43 (32.3)	34 (22.5)	117 (77.5)	0.11	0.06 – 0.21

comparison of the lowest and highest quartiles removed the impact of nuts on CHD risk (Table5). Association of nuts consumption was reported with decreased CHD in earlier prospective studies (**Dontas *et al.*, 2007**). In previous studies, it was found that consumption of at least 5 servings/wk of nuts or peanut butter was associated with lower LDL cholesterol, non-HDL cholesterol, and total cholesterol (**Li *et al.*,2009**). Nuts and peanuts contain many bioactive components which exert beneficial effects on these CHD risk factors (**Ros, 2009; Kris-Etherton *et al.*, 2008**).

In our study (Table 1) no significant difference was observed in average legume and whole grain intakes between individuals with and without CHD (case, 12.4 g/d vs. control, 14.7 g/d; cases, 25.3 g/d vs. controls, 30.0 g/d; respectively). In addition, the percentage of individuals in our study, who reported consuming less than 100 g/day of whole grain, was 95.9% and 93.4% of CHD patients and controls respectively. These findings are in contrast with other studies that indicated legume (**Bazzano *et al.*, 2001**) and whole grain (**www.plosone.org, 2012**) might have beneficial health influences to reduce the risk of CHD. It is remarkable to mention here that both legume and whole grain consumption was not considerable among the study population under study.

Although the cases had significantly lower intakes of total fish and seafood (16.0 g/d) compared to the controls (19.7 g/d), the low intake of fish (below 200 g/ week) was most common among cases (85%) and controls (81.3%).

In a Meta-analysis of cohort studies, compared with those who never consumed fish or ate fish less than once per month, individuals with a higher intake of fish had lower CHD mortality. Each 20-g/d increase in fish intake was related to a 7% lower risk of CHD mortality (P for trend=0.03) (Bazzano et al., 2004).

Given the objectives of our study, we conducted the analysis of plant and animal food consumption. In this study, controls had a significantly higher intake of plant food than the cases (791.3 g/ d vs. 625.0 g/d; P<0.0001). This was mainly due to the difference in the intakes of fruit and vegetable, while in stratified analyses for CHD, significant association was not observed when the bottom and top quintiles of animal foods consumption were compared. In contrast, in a case control study, which was conducted in Indonesia there was no difference in plant food intake between the two groups. Average intake of plant food was 1,061 g/d for the case group and 1,028 g/d in the control group. Also, the odds ratio for the subjects who consumed animal foods in the highest quartile (above 210 g) to those in the lowest quartile (below 108 grams) was 4.8 (95% CI 2.25-10.30, P<0.0001) (**Lipoeto *et al.*, 2004**), these differences may be related to food culture in this country. However, because of many other potential risk factors among diverse countries, dietary intake alone may not be the mere factor underlying lower CHD incidence.

5 Conclusion

Our finding revealed that fruit, vegetable, whole grain and plant food intakes independently were associated with CHD events. Thus these food groups could be predicted risk of CHD in this population. However, more studies are required to examine association dietary intake and CHD in Armenia.

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Outline of the 2011 off the Pacific Coast of Tohoku Earthquake (Mw 9.0) — Earthquake Early Earning and Observed Seismic Intensity

Mitsuyuki Hoshiba [1], Kazuhiro Iwakiri [1], Naoki Hayashimoto [1], Toshihiro Shimoyama [2], Kazuyuki Hirano [2], Yasuyuki Yamada [2], Yuzo Ishigaki [2], Haruyuki Kikuta [2]

[1] Meteorological Research Institute, Nagamine 1-1, Tsukuba 305-0031, Japan
[2] Japan Meteorological Agency, Ohtemachi 1-3-4, Chiyoda-ku, Tokyo 100-8122, Japan

ABSTRACT

The 2011 off the Pacific coast of Tohoku Earthquake (Mw 9.0) that occurred on March 11, 2011, caused strong ground motion around northeastern Japan. Before the strong ground motion hit cities, the Japan Meteorological Agency (JMA) issued Earthquake Early Warning (EEW) announcements to the general public of the Tohoku district and then the warning was automatically broadcast through TV, radios and cellular phone mails. The EEW was earlier than the S wave arrival and more than 15 s earlier than the strong ground motion (intensity 5-lower or greater on the JMA scale) everywhere in the district. Seismic intensity 7 was observed for only the second time since JMA introduced instrument-based observation for intensity measurements in 1996. Intensities of 6-upper and 6-lower were widely observed at many stations in the Tohoku and Kanto districts, over an area of approximately 400 km × 100 km. The duration of strong ground motions was quite long. For the Tokyo region, JMA EEW expected intensities of 4, which was an underestimation of the observed intensity (5-upper). This underestimation can probably be attributed to the large extent of the fault rupture.

Key Words

The 2011 off the Pacific coast of Tohoku earthquake, Strong ground motion, Seismic intensity, Earthquake early warning.

Correspondence to:

M Hoshiba

E-mail: mhoshiba@mri-jma.go.jp

1 Introduction

The 2011 off the Pacific coast of Tohoku Earthquake (Mw 9.0) occurred off the pacific coast of Japan on March 11, 2011, and caused a huge tsunami that killed more than 12,000 people, and left more than 12,000 people missing (**Fire Disaster Management Agency, Japan, report of April 11, 2011**). Strong ground motion was also recorded across a wide area of northeastern Japan. Just before the strong ground motion hit cities in the Tohoku district (northeastern Honshu Island), the Japan Meteorological Agency (JMA) issued the Earthquake Early Warning (EEW) announcements to the general public in the district. Seismic intensity of 7 (JMA scale) was observed, for only the second time since JMA introduced instrument-based intensity observations in 1996 (Hoshiba et al., 2010). Seismic intensities of 6-upper and 6-lower were also widely observed in the Tohoku and Kanto (central eastern Honshu) districts.

This paper outlines the warnings of strong ground motion (that is, EEW) and observations of the seismic intensity for the Mw 9.0 earthquake.

2 Observation of Seismic Intensity and Operation of JMA EEW

In Japan, the JMA intensity scale is widely used to measure seismic intensity. Since 1996 this scale has been based on instrumental measurements in which not only the amplitude but also the frequency and duration of the shaking are considered (**JMA, 1996; Hoshiba *et al.*, 2010**). The 10-degree JMA intensity scale rounds off the instrumental intensity value to the integer. Intensities of 5 and 6 are divided into two degrees, namely 5-lower, 5-upper, 6-lower and 6-upper, respectively. Intensity 1 corresponds to ground motion that people can barely detect, and 7 is the upper limit. At present, seismic intensity is measured at more than 4,000 places throughout Japan by JMA, municipalities, local governments and the National Research Institute for Earth Science and Disaster Prevention (NIED). When an earthquake occurs, the intensity data are transmitted to JMA and summarized. The summary is broadcast through multiple media starting within two minutes after the earthquake, and it is updated as the data increase.

EEW aims at mitigating an earthquake disaster by giving people enough time to take appropriate safety measures in advance of strong shaking. It has been operational nationwide in Japan by JMA since October, 2007. For JMA EEW, the hypocenter is determined by a combination of several techniques (**Hoshiba *et al.*, 2008**), using approximately 1,100 stations from the JMA network and the Hi-net network of NIED; magnitude is mainly from maximum displacement amplitudes (**Kamigaichi *et al.*, 2009**). The JMA EEWs are divided into two grades: "forecast" and "warning" (**Hoshiba *et al.*, 2008; Kamigaichi *et al.*, 2009; Doi,**

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2010). The EEW "forecast" is issued to advanced users when events are estimated to be M 3.5 or larger, or when the expected seismic intensity is 3 or greater. In the "forecast", the regions are particularly specified where seismic intensity 4 or greater is expected. When intensity is expected to be 5-lower or greater at any observation station of the seismic intensity networks, "warning" is issued to the general public in regions where intensity 4 or greater is expected. The "warning" is broadcast in various ways, such as by TV, radio and cellular phone mails. JMA EEWs are updated as available data increases with elapsed time. Accordingly EEWs are issued repeatedly with improving accuracy. The "warning" is updated when the seismic intensity is expected to be 5-lower or greater in regions where the intensity was estimated to be less than 4 in the first "warning": that is, where new regions are subject to shaking above the threshold. Even when the update causes the expected intensity to fall below 5-lower at any stations, the "warning" is not canceled so as to avoid confusion if the expected intensity rises again. In the updated "warning", the newly added regions are described. At present operation, an update of the "warning" is given only when the elapsed time is less than 60 s from the first trigger, to avoid too late a warning due to the fluctuation of gradually increasing amplitude of later phases (e.g., M 5.8 events of May 8, 2008; JMA, 2008).

3 EEW during the Mw 9.0 Earthquake

The JMA EEW system was triggered for the Mw 9.0 earthquake when station OURI detected the initial P wave at 14:46:40.2, Japan Standard Time, March 11 (JMA, 2011a, b). The first EEW "forecast", the first of 15 announcements, was issued 5.4 s later. The magnitude at the time was estimated to be 4.3, because the waveform started with small amplitude, which was comparable to noise level for displacement (**Hoshiba,Iwakiri, 2011**). The small amplitude does not indicate that the initial rupture of the Mw 9.0 event is large, and does not suggest a large magnitude event. Figure 1 shows the sequence of hypocenter and magnitude updates for the earthquake. For the Mw 9.0, hypocenter of **Horiuchi *et al.*** (**2005**)'s technique was adopted using data from Hi-net, and the magnitude was estimated from the four stations shown in Fig. 1. By the fourth "forecast", 8.6 s after the first trigger, the magnitude was estimated to be 7.2 and seismic intensity was expected to be 5-lower for central Miyagi prefecture (around Sendai city) and the fourth "forecast" was a "warning" to the general public in the Tohoku district. Then it was automatically broadcast through TV, radios and cellular phone mails. NHK, a non-profit broadcasting company, broadcast it nationwide, and other TV companies did so locally. The "warning" was earlier than the S wave arrival (observed intensity was at most 2) and also 15 s earlier than the time that strong ground motion (intensity 5-lower) hit the closest station to the epicenter (Fig. 2). The animation of the performance is shown in the Meteorological Research Institute (2011). The reason the magnitude decreased during the fifth through seventh "forecast" (9.6–15.9 s after the first trigger) was small magnitude estimated at the second station, ICHINM, due to the small amplitude of the initial part of the event. By the issue of fifteenth "forecast", 116.8 s after the first trigger, the magnitude was estimated to be 8.1. This estimated magnitude is almost the upper limit of JMA displacement magnitude because of amplitude-magnitude saturation for events of Mw > 8. Note that the displacement magnitude is estimated from maximum displacement amplitude which corresponds to the outputs of seismometers having eigenperiod of 6 s, and the mainshock displacement magnitude is 8.4 in the unified hypocenter catalog of JMA (**Hirose *et al.*, 2011**).

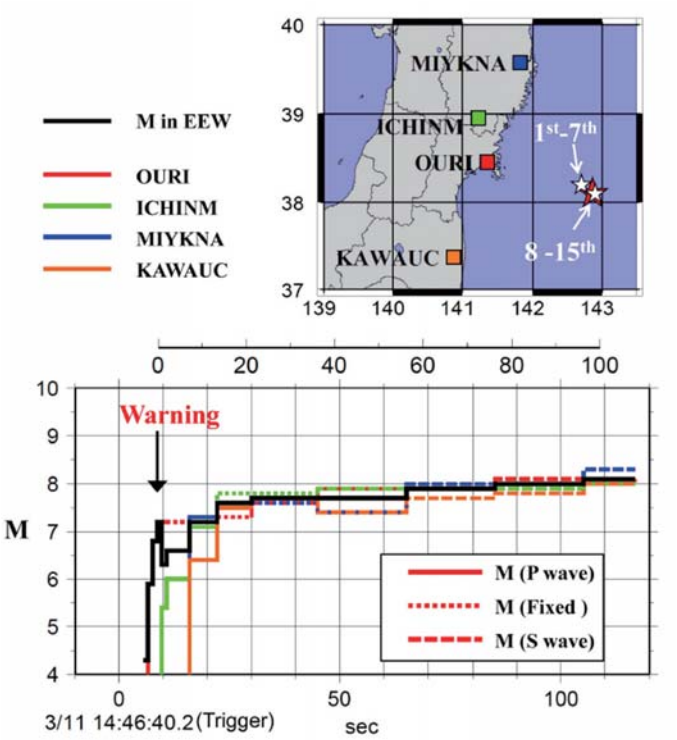

Fig.1 Sequence of determinations of epicenter and magnitude in JMA EEW. Upper right panel: epicenters determined by the EEW system are shown as a white star for the first to seventh "forecast" (5.4–11.0 s after the first trigger) and another for the next eight (15.9–116.8 s). Focal depth was estimated to be 10 km for all 15 announcements. Red star indicates the epicenter location from the unified JMA catalog (focal depth is 24 km). The resolution of the JMA EEW system is 0.1 degree for latitude and longitude, and 10 km for focal depth for hypocenter determination. Lower panel: magnitudes estimated from maximum displacement amplitude at four stations. Color lines represent the different stations; the black line is the median value, which is used for JMA EEW. Bottom axis shows the elapsed time from the first trigger, and offset axis at top shows time elapsed from the EEW "warning" (the fourth "forecast"). The solid line indicates the magnitude of the P wave, broken line is that of S wave, and dotted line shows the period during which the magnitude is kept unchanged around the S wave arrival.

4 Observed Seismic Intensity of the Mw 9.0

Figure 3 shows the distribution of the observed seismic intensities of the March 11 mainshock, and the 2003 Tokachi Oki earthquake (Mw 8.0). The intensity contours are based on the observed intensities from the dense seismic intensity networks after taking

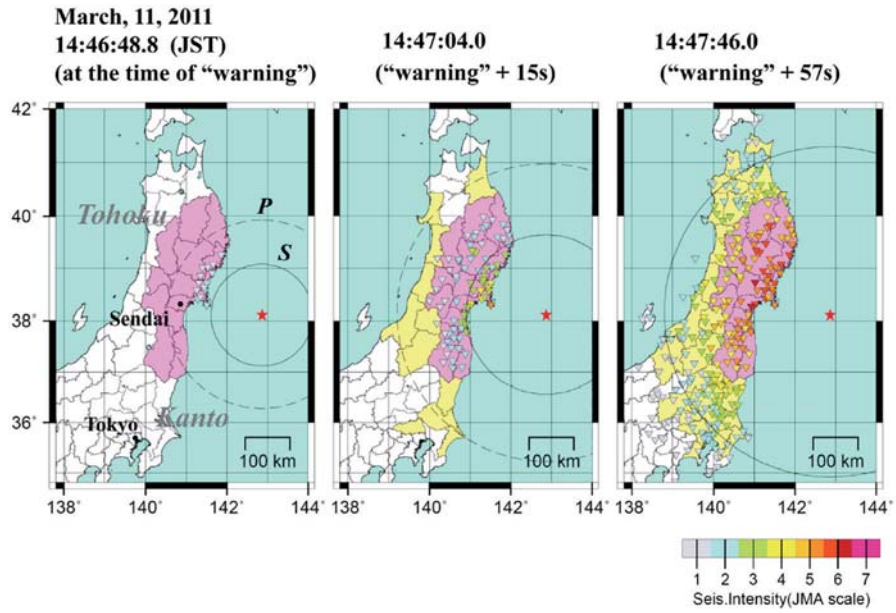

Fig.2 Region of EEW "warning" and "forecast", and distribution of seismic intensities estimated in real time manner (Kunugi et al., 2008) at 14:46:48.8 when the "warning" was issued (left), at 14:47:04.0 when intensity 5-lower or greater first appeared (center), and at 14:47:46.0 when the Tokyo region was first specified in the EEW "forecast" (right). Pink area indicates the region where the "warning" was issued, and the yellow areas are those specified in the "forecast". Wave fronts of P and S waves are shown by broken and solid circles, respectively. The seismic intensities (colored triangles) were measured using waveforms of the K-NET, KiK-net (NIED), and JMA networks. The animation of this figure is shown in the Meteorological Research Institute (2011). The distribution of the eventual seismic intensity, which means finally observed intensity, is shown in Fig. 3.

into account the site amplification factors, which is similar to the idea of ShakeMap (**Wald *et al.*, 2006**). The area of strong ground motion (6-upper and 6-lower) extends from the Tohoku to the Kanto district, over an area of approximately 400 km × 100 km, which is much larger than the corresponding area of the 2003 Tokachi Oki earthquake. K-NET station MYG004 in Kurihara city, at which intensity 7 was recorded, is 175 km from the epicenter, and some stations at which 6-lower intensity were observed in the Kanto district are more than 350 km from the epicenter.

In addition to the wide area of strong ground motion, a long duration was characteristic of the event. Figure 4 shows the acceleration at stations OURI (relatively near the epicenter, 138 km) and IYASAT(relatively far, 315 km), along with the seismic intensity measured in real-time manner using Kunugi et al.'s (2008) method. For comparison, acceleration and intensity at KiK-net station IWTH25 are also shown at the time of the 2008 Iwate-Miyagi Nairiku earthquake (Mw 7.0, 3 km). At OURI, two peaks are apparent in the acceleration envelope, which is probably due to the complicated source process. The second peak was 50 s after the first peak. At IYASAT, strong ground motion was apparent from later phases, well after the direct S phase, and it took 80 s to increase from intensity 1 (barely felt) to 5-lower, the level at which most people were frightened. The duration is very long as compared to 1 s which was observed at IWTH25 at the Mw 7.0 event. Ground motions corresponding to intensity 4, or greater, continued for 120 to 190 s at many observation stations in the Tohoku and Kanto districts (JMA, 2011c).

The EEW system expected intensity of 4 in the Tokyo region in the twelfth to fifteenth (final) issues (Fig. 2). This was an underestimation. Actual observations reached 5-upper, which is greater than the criterion of the EEW "warning". The underestimation can probably be attributed to the large extent of the later fault rupture. For the northern part of Ibaraki prefecture (around IYASAT), where intensity expected in the first warning (fourth "forecast") was less than 4, the expected intensity rose to 5-lower by the fourteenth "forecast", but it was too late to update the "warning", because it was issued 105 s after the trigger, which is later than the 60 s criteria at which upgrades are stopped.

5 Summary and Remarks

The 2011 Tohoku Earthquake (Mw 9.0) generated widespread strong ground motion, and seismic intensities of 6-lower and 6-upper were recorded in the Tohoku and Kanto districts over an area of approximately 400 km × 100 km. The durations of strong ground motion were very long.

The JMA EEW system issued one "warning" to the general public in the Tohoku district before the start of strong ground motion. It was earlier than the S wave arrival and 15 s earlier than strong ground motion (intensity 5-lower or greater) at the closest station to the epicenter.

After the mainshock, the EEW system did not work well for several

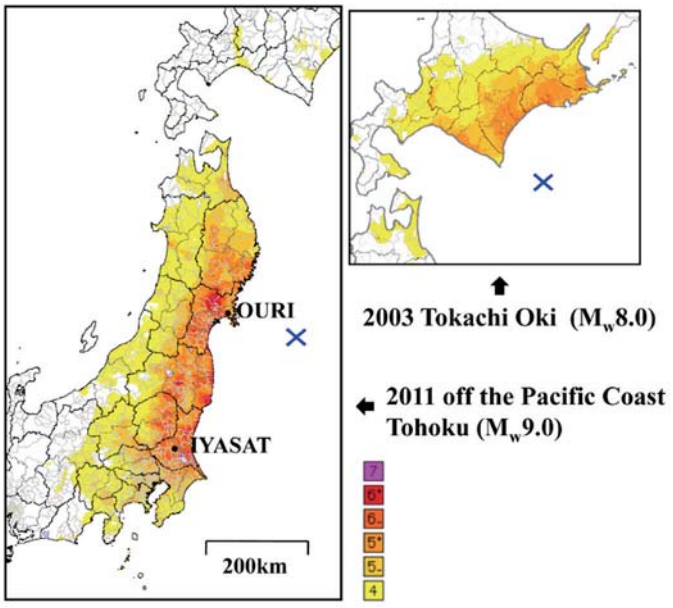

Fig.3 Seismic intensity distribution of the 2011 Mw 9.0 earthquake and the 2003 Tokachi Oki earthquake (Mw 8.0).

hours because of high background noise from the coda waves of the mainshock and active aftershocks, and because of power failure and wiring disconnections. For several days, when earthquakes sometimes occurred simultaneously over the wide source region, the system became confused, and did not always determine the location and magnitude correctly. In 19 days from the mainshock to March 29, 2011, JMA appropriately issued EEW "warning" for 15 of the 22 events for which seismic intensity 5-lower or greater was actually observed. On the other hand, during the same time, 45 EEW "warnings" were issued, but actual observed intensities did not exceed 2 at any observation stations in 11 of the 45 events (JMA, 2011d).

Acknowledgement

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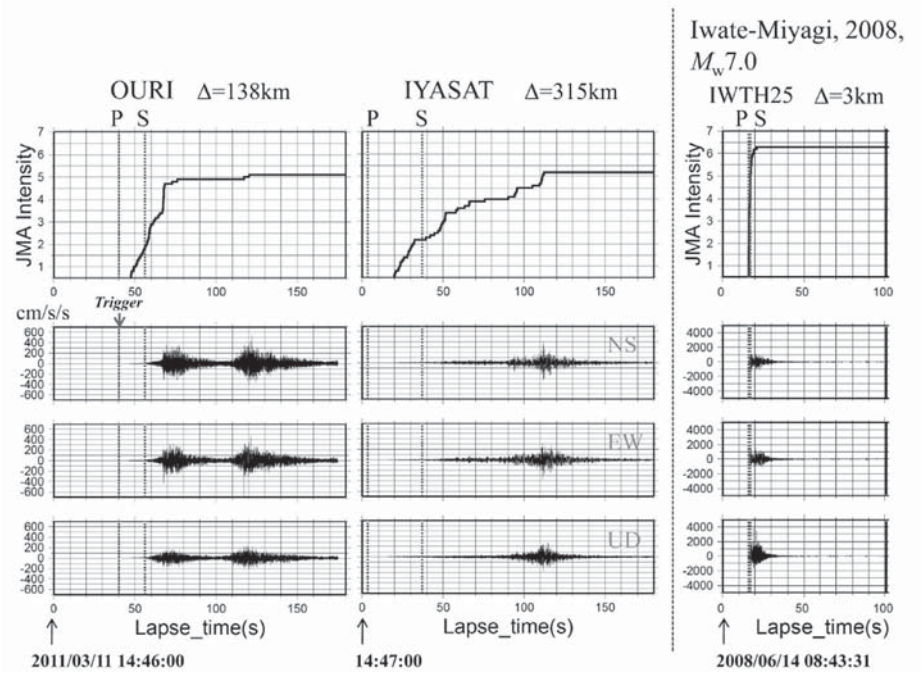

Fig.4 Acceleration waveform and seismic intensity estimated in real-time manner at OURI and IYASAT. P and S wave arrival times are shown by dotted lines. The arrow labeled "Trigger" indicates 14:46:40.2, when station OURI triggered the EEW procedure. For comparison, acceleration and intensity at IWTH25 (KiK-net) are also shown at the time of the 2008 Iwate Miyagi Nairiku earthquake (June 14, 2008, focal depth is 8 km, Mw 7.0).

Lipidomics: A New Window to Biomedical Frontiers

Matej Orešič [1,2], Virve A. Hänninen [3], Antonio Vidal-Puig [4]

[1] VTT Technical Research Centre of Finland, P.O. Box 1000, Espoo, FI-02044 VTT, Finland
[2] Institute of Molecular Medicine Finland FIMM, P.O. Box 20, Helsinki, FI-00014 University of Helsinki, Finland
[3] Department of Ophthalmology, Helsinki University Central Hospital, P.O. Box 220, Helsinki, FI-00029 HUS, Finland
[4] Metabolic Research Laboratories, Institute of Metabolic Science, Addenbrooke's Hospital, University of Cambridge, Cambridge CB2 0QQ, UK

ABSTRACT

Lipids are a highly diverse class of molecules with crucial roles in cellular energy storage, structure and signaling. Lipid homeostasis is fundamental to maintain health, and lipid defects are central to the pathogenesis of important and devastating diseases. Newly emerging advances have facilitated the development of so-called lipidomics technologies and offer an opportunity to elucidate the mechanisms leading to disease. Furthermore, these advances also provide the tools to unravel the complexity of the 'allostatic forces' that allow maintenance of normal cellular/tissue phenotypes through the application of bioenergetically inefficient adaptive mechanisms. An alternative strategy is to focus on tissues with limited allostatic capacity, such as the eye, that could be used as readouts of metabolic stress over time. Identification of these allostatic mechanisms and pathological 'scares' might provide a window to unknown pathogenic mechanisms, as well as facilitate identification of early biomarkers of disease.

Correspondence to:

Orešič M

E-mail: matej.oresic@vtt.fi

Introduction

Lipids are a diverse group of compounds with many key roles enabling them to serve as forms of energy storage, structural moieties and signaling molecules [1] (Figure 1). Lipids are broadly defined as hydrophobic or amphiphilic small molecules that originate either entirely or in part from two distinct types of building blocks: ketoacyl and isoprene groups [2]. Lipids are structurally highly diverse owing to the many possible variations of the lipid building blocks and how these blocks are linked. We have conservatively estimated that the theoretical number of lipids covering major lipid classes is close to 200 000 [3]. Lipids are very abundant in biological systems, constitute ~50% of the mass of most animal cell membranes[4] and exhibit an important degree of specialization in specific cellular compartments [5]. Maintenance of an appropriate lipid composition in the cellular membranes is required to ensure membrane fluidity, topology of attached proteins, activity of membrane-bound enzymes, degree of exposure of surface proteins, lateral mobility of receptors and activation of specific signaling pathways. Interestingly, despite being exposed to the diverse nutritional composition of organismal diets membranes maintain a remarkable control of their lipid composition, which reinforces the concept that specific mechanisms exist to ensure the maintenance of the homeostasis of the lipid composition of membranes.

The development of a pathological state represents a situation of biological stress associated with the breakdown and failure of the mechanisms controlling homeostasis. Given the tight homeostatic control of lipid metabolism and its enormous structural and bioenergetic relevance it is not surprising that alterations in lipid metabolism play important roles in the pathogenesis of most of the common diseases, including insulin-resistant diabetes [6,7], Alzheimer's disease [8,9], schizophrenia [10,11], cancer[12,13], atherosclerosis [14] and toxic manifestations of infectious diseases[15,16]. It is interesting to note that some of these apparently diverse diseases tend to cluster together in the same individuals, a phenomenon known as the metabolic syndrome (MetS) [17].

The prevalence of the MetS, defined as the coincidence in the same individual of obesity, insulin resistance, dyslipidemia, hypertension and increased cardiovascular morbidity, is increasing exponentially within several populations worldwide, with a profile that could be considered of epidemic proportions[18]. This profile clearly indicates that in addition to undeniable genetic contribution, other environmental factors, such as nutrients and inactivity, are likely contributors to the progressive acceleration of MetS. Despite its obvious public health and economic implications and worldwide research efforts, the sad truth is that the molecular mechanisms linking all these manifestations are still

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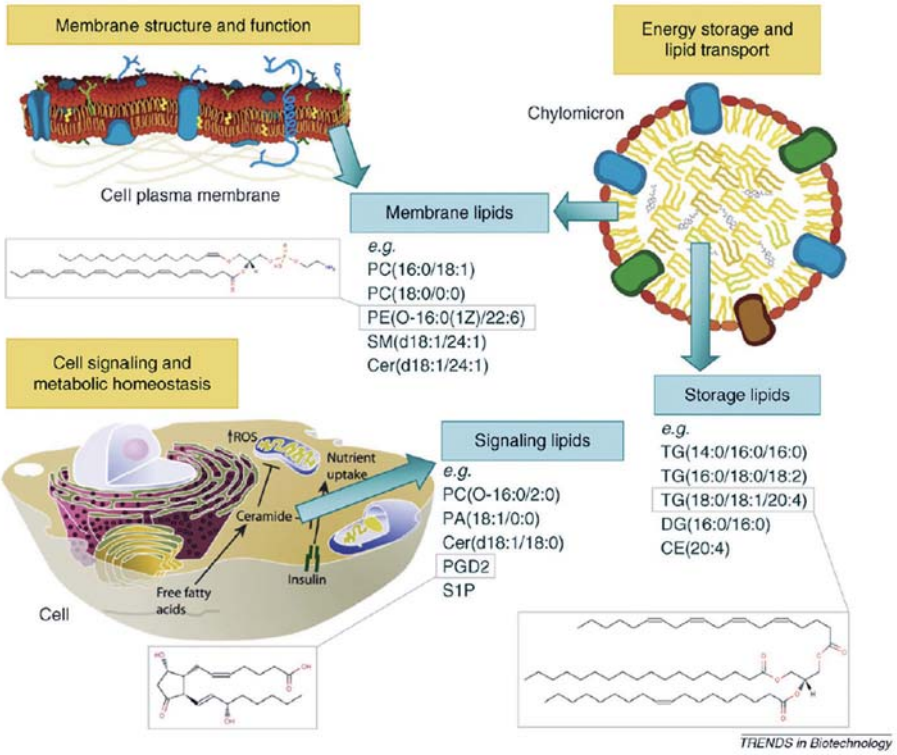

Fig.1 Diverse biological roles of lipids, with a few common representative molecular species listed. The chemical structures are shown for ethanolamine plasmalogen PE (O-16:0 (1Z) / 22:6), prostaglandin D2 (PGD2) and triacylglycerol TG (18:0 / 18:1 / 20:4). PC, phosphatidylcholine; PE, phosphatidylethanolamine; PA, phosphatidic acid; DG, diacylglycerol; Cer, ceramide; SM, sphingomyelin; ChoE, cholesterol ester; S1P, sphingosine-1-phosphate.

elusive; in part because of the difficulty in discriminating primary pathogenic events from secondary allostatic responses. However, in recent years, accumulating evidence suggests that lipids, and more specifically their toxic effects when they excessively accumulate in areas other than the adipose tissue, might provide vital clues to understand the pathogenesis of the MetS [19]. The key concept of a pathogenic framework is lipotoxicity [20,21]; the toxicity produced by lipids that cause insulin resistance when accumulated in metabolically relevant organs such as skeletal muscle, liver, pancreatic b cell or vascular wall. In those organs, the accumulation of lipids not only distorts specific signaling pathways, such as insulin action and or other growth factors, but also induces important allostatic responses in an attempt to maintain energy homeostasis.

Traditional approaches to the study of lipids have relied on analyses that were specific to a lipid class, or to a particular fatty acid composition, and thus have likely addressed only a small fraction of the lipid complexity important in the physiological context. Here we suggest that the new technology platforms and bioinformatics tools that are becoming available for the study of lipids offer an opportunity to provide a sensitive readout of integrated metabolic stress over time, as well as to unravel the complexity of the allostatic forces that allow maintaining normal phenotypes at the expense of highly expensive adaptive mechanisms (Box 1). Identification of these allostatic mechanisms and pathological insults might provide an invaluable window to novel pathogenic mechanisms as well as helping to identify early diagnostic and prognostic biomarkers of complex diseases.

The emergence of lipidomics

Owing to their central biological role, lipids have been anintense area of research since the 1960s, yet since that era have been somewhat overshadowed by the advent of molecular biology, genomics and proteomics. The major bottleneck in lipid research has been the unavailability of sensitive analytical platforms capable of detecting the intact lipid molecular species in specific biological systems.

Glossary

Allostasis: allostasis is the process of achieving stability, or homeostasis, through physiological or behavioral change (Box 1).
Aqueous humor: a thick watery substance located between the lens and the cornea (Figure 2a).
Avascular: not associated with or supplied by blood vessels.
Biomarker: a substance used as an indicator of a biologic state. It is a characteristic that can be objectively measured and evaluated as an indicator of normal biologic processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention.
Effector: a molecule that activates, controls, or inactivates a biological process or action.
Glycemia: concentration of glucose in the blood.
Homeostasis: the property of a living organism that regulates its internal environment so as to maintain a stable, constant condition.
Hyperglycemia (or high blood sugar): a condition in which an excessive amount of glucose circulates in the blood plasma.
Hypoxia: a shortage of oxygen.
Lipotoxicity: accumulation of (lipo) toxic reactive lipids such as ceramides in nonadipose tissues of metabolically important organs, such as pancreas, skeletal muscle, liver, and heart.
Nuclear cataract: a clouding that develops in the nucleus, the center of the eye lens. It can vary in degree from slight to complete opacity and obstruct the passage of light.
Prodromal period: the time during which a disease process has already begun, but is not yet clinically manifest.

Additionally, owing to their tight homeostatic control, even relatively small changes in the concentration of lipids –of the order of ±10 % – can be physiologically significant. Thus, the study of lipids in a biomedical context requires the availability of platforms that can accurately and comprehensively quantify the lipids. The advent of modern analytical techniques for metabolomics[22,23], empowering us with the ability to detect and quantify hundreds of intact lipid molecular species in parallel, has thus rejuvenated the field of lipid research over the past decade and has led to emergence of lipidomics [24–26] (Box 2).

Owing to these technological advances, lipidomics is currently at the stage where vast amounts of data are

Box 1 Allostasis

The concept of allostasis refers to maintenance of stability through change. This concept could be applied to multiple fields spanning from sociological networks, animal behavior, or more relevant for this Opinion, to metabolic diseases. The concept of allostasis was introduced by Sterling and Eyre to describe the adaptive mechanisms that allow maintenance of normality at the expense of robust, energy-costing adaptive mechanisms [33]. Whereas the concept of homeostasis refers to mechanisms that prevent change and ensure the maintenance of the ideal steady state of a function (e.g. pH, dissolved oxygen), allostasis refers to the changes that are required in response to severe challenges and are aimed at restoring the homeostasis of the system. Working under allostatic conditions is expensive, and the cost has been defined as the allostatic load of a system. The allostatic load refers to the cumulative stress derived from the activation of adaptive changes to maintain the functionality of the system. Allostatic load is proportional to the inefficiency of the allostatic mechanisms and to the intensity of the challenge. Depending on the strength or conversely the fragility of a biological system, the allostatic load can be either tolerated or not. In particularly vulnerable biological systems, the allostatic load results in allostatic overload which is proportional to the risk of global failure of the system, or in biomedical terms, to the development of disease. Contributing factors to the allostatic load are the organ specific genetic make up, the environmental experiences during early development, cultural impact on life style choices with regard to diet, exercise, smoking and drinking. All of these factors influence the charge of the allostatic reactivity of the biological system. Thus allostatic load reflects a genetically or developmentally programmed inefficiency to respond to environmental challenges.

becoming available, yet the tools and the knowledge needed to interpret these data in their biological context are lacking to a large extent. The particular difficulty of a bioinformatic analysis of data obtained from lipidomics stems from the fact that the measured lipid concentrations reflect regulation at multiple spatial and dynamic scales, for example global changes in cell membrane composition, systemic lipid metabolism, lipid trafficking, lipid oxidation or biochemical reactions. Even at the biochemical level, available metabolic pathway databases such as KEGG (Kyoto Encyclopedia of Genes and Genomes) [27] do not capture lipids and their reactions with the degree of structural detail that can be detected with the current lipidomics technologies[28,29]. New tools are therefore needed to decipher the obtainable information on lipids at the molecular level. The LIPID MAPS consortium (LIPID Metabolites And Pathways Strategy; http://www.lipidmaps.org/) recently proposed a new naming system for lipids [2] that assigns a unique 12-character signature for biologically relevant lipids. This unique identification number for a lipid provides valuable information, such as source of database, lipid category, class, subclass and numbers specific to the particular lipid. The LIPID MAPS system therefore enables to efficiently store and process information on a vast amount of different lipids. This new nomenclature system is expected to further the field because it enables automated processing of lipid information, which is needed for dealing with vast amounts of data being generated by lipidomics platforms.

To address the problem of lipid pathway mapping at the molecular level, we recently developed a lipid pathway instantiation strategy [28]. Using this approach, the global lipidomics data are first analyzed using multivariate statistical methods. Clusters of lipids that are co-regulated under specific physiological conditions are so identified, and biochemical pathways can then be reconstructed for specific lipids from the clusters of interest. Future strategies for modeling of lipidomics data will probably require the combination of multiple biocomputational strategies, such as pathway reconstruction [28], modeling of systemic lipid metabolism [30,31] and biophysical modeling [32].

Box 2. Lipidomics technologies in brief

The analytical strategies for the study of a lipidome can be divided into two overlapping categories: global and targeted lipidomics.

(1) Global lipidomics aims at rapid identification and relative quantification of hundreds of molecular lipids across multiple structural classes, usually sourced from the total lipid extracts. The global strategies include 'shotgun lipidomics,' which uses direct infusion of lipid extracts into a mass spectrometer [48,49], as well as liquid chromatography coupled to mass spectrometry (LC–MS) [50,51].

(2) Targeted lipidomics aims at quantitative analysis of either a single or several selected lipids, e.g. within a specific lipid class [52,53].

Data processing, which consists of peak detection, spectral alignment, normalization, and identification, is an essential part of global and quantitative lipidomics platforms. Software packages used for metabolomics data processing, such as MZmine [54] or XCMS [55], have been commonly applied to LC–MS-based approaches. Specialized lipidomics software solutions have also been developed for specific analytical strategies [56,57]. Among the many exciting technological developments in lipidomics, the ozone-induced dissociation (OzID) [58] technique promises to solve the problem of an exact structural determination of numerous lipids by determination of the position of the double bonds. Double bond positioning is particularly important in structural elucidation of plasmalogens, i.e. the endogenous antioxidants [59], or for distinguishing closely related fatty acids within lipid molecules of the same mass and functional class, e.g. the v-3 and v-6 fatty acids.

Lipids: a physiological view

The maintenance of the membrane lipid composition despite nutritional and other environmental stresses constitutes a good example of an allostatic change, which as first introduced by Sterling and Eyer [33] is defined as an adaptive process aimed at effectively maintaining stability through changes in specific regulatory mechanisms. Typically, such allostatic adaptations are designed to induce short-term corrective changes to regulatory systems. However, when activated for long periods, for example during chronic states of overnutrition or undernutrition, the maintenance of lipid homeostasis might actually be achieved at the expense of a metabolic cost, or 'collateral damage,' defined by McEwen as allostatic load [34–36]. For instance, maintenance of allostatic changes might induce permanent changes in the default setting of the biological system that could prevent an appropriate shut down after the stimulus has been discontinued. Another undesired effect of allostatic load might the inappropriate activation of alternative biosynthetic pathways, which could lead to the accumulation of

unnecessary lipids.

There is evidence that the maintenance of lipid membrane composition is tightly regulated by complex mechanisms involving several transcription factors (TFs), such as sterol regulatory element binding proteins (SREBPs), whose activity is further modulated by sensitive sensory systems in response to changes in lipid levels[37,38]. Interestingly, it has been observed that genetic ablation of the gene encoding SREBP1, the TF that regulates preferentially fatty acid metabolism, is compensated by upregulation of SREBP2, the TF preferentially controlling cholesterol metabolism, with the consequence that inappropriate amounts of cholesterol are accumulated. This on the one hand can be interpreted as an example of the tightness of the control in lipid metabolism, but on the other hand can serve as an example of collateral damage caused or allostatic load produced through an inappropriate activation of the cholesterol pathway in an attempt to maintain the homeostasis of fatty acids.

Thus, the allostatic load derived from the allostatic adaptations, together with a specific genetic make-up and in the context of specific developmental stages (e.g. early-life experience, puberty, pregnancy, menopause, aging), metabolic status (e.g. obesity) or bioenergetic demands of an individual, might define different degrees of vulnerability to disease. In the course of our research, we have observed that, although these lipid-related allostatic mechanisms can use similar effectors, they might involve different pathways in different organs that are defined by their specific genetic repertoire. This confers an additional layer of complexity to the analysis of lipids, but by contrast opens new opportunities for selective interventions to ease the allostatic load [7, 28, 39].

Despite the obvious biological importance of allostatic loads, there is a paucity of data for the underlying allostatic mechanisms that ensure the maintenance of the cell membrane lipid homeostasis, and how these mechanisms are adapted to specific nutritional and metabolic perturbations. The recent advances in lipidomics technology offer a unique opportunity not only for the biochemical characterization of lipids but also for a more comprehensive understanding of the influence lipids have on a biological system, such as their effects on cellular membranes with respect to membrane architecture, transcriptional and translational modulation, cell signaling, cell–cell interaction and response to environmental change over time. Given the nonlinearity of the allostatic mechanisms, the main challenge is to determine how the different effectors of the allostatic response are modulated dynamically with the level of change of each of the effectors. In this regard, lipidomic analysis in the context of a systems-biology approach offers an unprecedented opportunity to elucidate the complexity of the allostatic mechanisms involved in maintaining lipid homeostasis.

Eye lipidome: a window to the past

The lipidome of an organism varies with gender, age and lifestyle. In early childhood, multiple changes in the constitution of circulating lipids reflect rapid developmental and environmental changes [40]. In older individuals, the lipidome is characterized by a decrease in the proportion of antioxidant lipids, thus reflecting the increase of oxidative stress with age [41]. One of the main challenges the healthcare industry faces is to be able to develop and apply effective preventive medicine. For this, early diagnostic and prognostic biomarkers are required to identify those individuals who will benefit the most from early intervention. Most common diseases are preceded by a long prodromal phase during which no objective symptoms are evident. For example, development of diabetes towards common forms of type 2 diabetes is preceded by a variable period during which insulin resistance is appropriately compensated by the secretion of the hormone insulin to maintain normal glycemia [42]. It is currently unclear which individuals amongst patients will evolve towards overt diabetes, or who will exhibit a severe cardiovascular outcome. The occurrence of visible symptoms, such as hyperglycemia or the development of atherosclerotic lesions, are late events in the progress towards the symptomatic disease. By contrast, we believe that there is an invaluable opportunity to use some of the early allostatic responses as markers of an allostatic load, which could be correlated to a quantified risk of disease progression.

An alternative to the use of allostatic responses as biomarkers could be a focus on tissues and organs with decreased allostatic capacity that, in this respect, are more suitable to record the 'scares' of the pathological insult. On one hand, from a Darwinian point of view, it is obvious that important metabolic organs, which are under pressure to perform regular and essential metabolic functions, are likely to have been selected to adapt more rapidly and fully, thus making it more difficult to detect early pathogenic mechanisms or past insults. On the other hand, metabolically less active organs, but with a specialized function, such as the lens, which is avascular and needs to maintain transparency, tend to be deprived of the common allostatic adaptive mechanisms[43] (Figure 2a). In this regard, the lens can become a reporter of integrated metabolic stress over time.

In the lens, the progressive accumulation of sphingolipids might cause the lipid hydrocarbon chain regions to become more ordered with age and cataract, which lowers its vulnerability to oxidative damage. Probably partly due to its diminished allostatic control, the lens lipid composition is tightly controlled evolutionarily. In fact, the lifespan of many species has been found to positively correlate with the sphingolipid content in the lens (Figure 2b), and inversely correlate with the lens phosphatidylcholine, which is known to be more prone to oxidative damage [44].

The lens obtains most nutrients by diffusion from aqueous humor and metabolites must often diffuse over long distances to reach the lens cells. Another relevant aspect of lens metabolisms is

the hypoxic environment around the lens, which appears to regulate the rate of lens growth and might also be important for protection against nuclear cataracts. Interestingly, lipoproteins are present in aqueous humor [45] and might undergo oxidation in certain systemic diseases, including renal failure, type 2 diabetes, atherosclerosis, inflammation and aging. We therefore predict that thorough studies of lens lipids and their surrounding fluids with modern lipidomics techniques [46] could be a novel approach able to identify novel early pathogenic insights or biomarkers related to multiple complex diseases that are characterized by long prodromal periods.

The currently used mass spectrometry techniques for lipidomic analysis demand complex sample preparation before analysis and are thus not suitable for the noninvasive determination of lens lipid composition in vivo. A promising technique, Fourier transform near infrared spectroscopy (FT-NIR), has recently been adapted to enable noninvasive measurement of body fat [47]. Although less sensitive and selective than mass spectrometry based methods, the use of optical techniques such as FT-NIR could constitute a feasible strategy for biomarker screening of lens lipids in a clinical setting. Complementarily, mass spectrometry based methods could be applied to study eye lipidomes in nonclinical studies, as well as to accurately quantify lipids in eye fluids, such as in aqueous humor.

Concluding remarks

There is evidence that lipid homeostasis is fundamental to maintain health and that defects in lipid metabolism and homeostasis are involved in the pathogenesis of important and devastating diseases. However, there has been a paucity of knowledge with regard to the role of lipids compared with that of other more easily addressable fields, such as genetics or protein chemistry. This lack of information is not the result of its irrelevance, but in fact the result of the intrinsic difficulty to quantitatively analyze lipids. For many years, the bottleneck of lipid research was the lack of highly sensitive analytical platforms able to provide accurate identification and quantification of lipids with roles in health and disease. The recent development of lipidomic platforms together with biocomputational tools to model metabolism and disease pathways offer an unprecedented opportunity to overcome this bottleneck. The power of the new profiling technologies linked to improved modeling tools offers an unprecedented opportunity to unravel the complexity of the allostatic forces that allow to maintain normal phenotypes at the expense of costly adaptive mechanisms. We have argued here that these adaptive mechanisms, besides their role in constituting potential therapeutic targets, might also become suitable early markers of a pathogenic insult. Alternatively, we suggest that a focus on tissues and organs with decreased allostatic capacity, such as the lens, might provide a sensitive readout of integrated metabolic stress suffered over time. Identification of these allostatic mechanisms and pathological insults might provide an invaluable window to novel pathogenic mechanisms as well as serve to provide early diagnostic and prognostic biomarkers of disease.

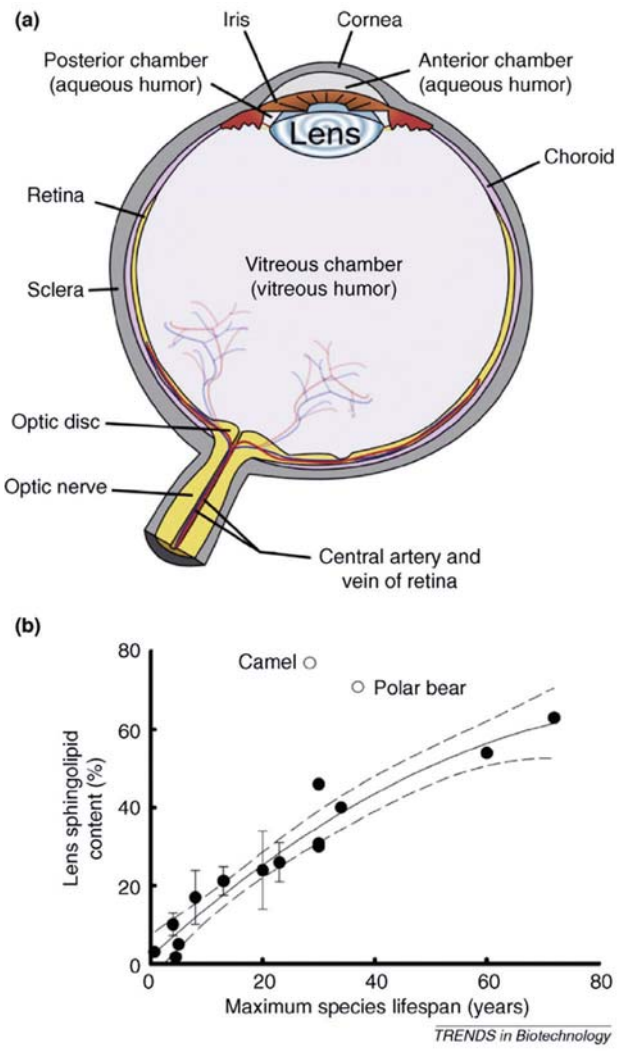

Fig.2 (a) Schematic diagram of the human eye. Only the lens and cornea are avascular tissues under nonpathological conditions. (b) Relationship between differences in lens lipid composition and maximum lifespan of a species, as adopted with permission from Borchman et al. [44]. Each dot in the diagram represents a different species, ranging from roach (0.7 year) and rabbit (4 years) to elephant (60 years) and human (72 years). The horizontal axis represents the maximum lifespan of this species in years and on the vertical axis the percent sphingolipid composition of the lens is shown. The center line indicates the linear regression curve fit with an order of two and the outer lines indicate the 95% confidence limits. Error bars are the standard error of the mean. The figure clearly demonstrates that lens sphingolipid content is related to the maximum lifespan of a species. The lenses of the polar bear and camel have exceptionally high levels of sphingolipids, which is likely due to living in adverse conditions [44].

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Research on Robbery Offenders' Attention to Negative Emotion

YANG Dong *, [1], LIU Zicheng [2], PENG Min [2]

[1] School of Psychology, Southwest University, Chongqing 400715, China
[2] Department of Criminal Technology, Interpol Corps, Public Security Bureau of Chongqing, Chongqing 400021, China

ABSTRACT
From the paradigm of visual search, this essay discusses the characteristics of attentional bias of robbers toward emotions (angry faces and happy faces). Data shows that, compared with the non-violent group, the violent group's reaction time toward angry faces is obviously shorter than happy faces, which preliminarily verifies the attentional bias of robbery offenders toward negative emotional information.

Key Words
Robbery offenders, Attentional bias, Emotional faces.

Correspondence to:

Assoc. Prof. YANG Dong
E-mail: 275538442@qq.com

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1 Preface

Crimes committed by robbery offenders is a prominent problem in modern society which brings about destructive effect on the individual and its family, and is harmful to social stability and harmony, thus winning attention from all parties. However, previous researches on crimes committed by robbery offenders have centered upon the victims of crimes committed by robbery offenders, and little research has been made directly into crimes committed by robbery offenders. In order to look into the causes of crimes committed by robbery offenders, we must have an in-depth understanding of the robbery offenders who are the executors of crimes committed by robbery offenders.

Presently, researches on the highly aggressive group of robbery offenders have centered on personality characteristics (**LI Xuemei, KUANG Li, AI Ming, CHEN Jianmei, LI Daqi & GAO Xinxue, 2008; LI Baohua, WANG Bin, ZHANG Jinxiang, ZHANG Zeng, LIU Guixian & HU Junmei, 2010**), such as A-type personality, trait anger and trait aggressiveness. Some researches have found that, compared with low performance motivation, subjects of of A-type personality are more prone to pay attention to such words as anger, hostility and aggressiveness under the high performance motivation (**Faunce, Mapledoram & Job,2004**). And there are other researches involving the effect of school, family and society on robbery offenders (**LI Baohua, WANG Bin, ZHANG Jinxiang, ZHANG Zeng, LIU Guixian & HU Junmei, 2010; ZOU Zhili, MENG Huaqing, Hu Hua, WANG Hui, LIANG Huaping & DU Lian et al, 2011**). Nevertheless, at present, little research has been made to discuss the cognitive mechanism of robbery offenders. One argumentation considers that robbery offenders may have defects in cognitive function, for example, Teichner et al find that the cognitive function of batterers is obviously inferior to normal subjects (**Teichner et al., 2001**). Laboratory researches have also found that violent torturers with anti-social personality are unable to accurately identify the facial expressions of others (**Babcock *et al.*, 2008**). What's more, some researchers have found that robbery offenders may make all kinds of wrong cognitive decisions, for instance, they usually make subjective guess and conjecture in the event of absence of objective basis; infer a rule out of a particular event, and extend it to obviously-unsuitable circumstances; exaggerate the importance of some events; and wrongfully attribute the reasons of some events to malicious attack of others (**Eckhardt *et al.*, 1998**). Despite that we are not clear about the reasons causing those defects in cognitive function, a possible explanation is that, in social circumstances, robbery offenders are prone to put excessive attentional resources into negative stimulus, resulting in their behavioral response that is inopportune. This attentional bias is also found in anxiety neurosis and some other groups of people (**Williams *et al.*, 1996; Jansen *et al.*, 2005**). Attentional bias means that, in relation to neutral stimulus, an individual allots attentional resources to some threats that affect the existence of human beings or similar stimulus carrying unsecure factors in

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priority (**Bar-Haim, Lamy, Pergamin, Bakermans-Kranenburg, & van Ijzendoorn, 2007; MacLeod, Mathews, & Tata, 1986; Mogg, Bradley, Hyare, & Lee, 1998**). In laboratory research, we usually use angry pictures to represent threat stimulus, since the people generally think that angry faces carry threat signals, which may be the reason why an individual processes angry faces faster and more efficient (**Vuilleumier & Schwartz, 2001**), which is mainly manifested in the fact that angry faces can preferentially attract more attentional resources (e.g., **Pratto & John,1991**). Researchers define this individual's ability of perception of angry faces faster and more efficient than that of happy faces as angry ex syndrome (AES). This ex syndrome is the product of evolution of human individual, and the instinct response of a normal individual in face of threat stimulus. Because the special group of people of robbery offenders frequently engage in extremely negative events, such as killing, ill-treatment and robbery, will robbery offenders have attentional bias toward negative stimulus and be more sensitive to it?

This study takes the highly aggressive group of people of robbery offenders as the object of research, and non-robbery offenders as the group of cross reference, and selects oriental emotional faces with localized characteristics rather than abstract emotional words as stimulus materials, and in the meanwhile, select some common emotional faces in interpersonal circumstances, i.e. angry face, happy face and neutral face, to examine the cognitive characteristics of robbery offenders, so as to better understand all kinds of violent behaviors, and provide some basis for preventing and correcting crimes.

2 Research Approaches

2.1 Subjects

We select 13 robbery offenders and 13 non-robbery offenders under custody at a house of detention in Chongqing municipality of China, all of whom are male. According to the standard of classification, we select robbery offenders covering murder, kidnapping, robbery, fire-raising and rape (**Smith & Waterman, 2004**), of age between 16 and 31, and 25 on the average, and SD=7.071; non-robbery offenders covering drug trafficking and property crimes, of age between 16 and 36, and 23 on the average, and SD=4.215. All of them are dextromanual, having normal vision or corrected vision acuity, and free from color blindness or weakness. Three of the subjects are rejected in data analysis due to heavy EEG artifact, so there are only 23 valid subjects, including 12 robbery offenders and 11 non-robbery offenders.

All subjects are voluntary and have signed the Informed Consent Form for Experimental Participants, and have the right to leave at any time during the experiment.

2.2 Experimental Materials and Instruments

In order to improve the ecological validity of the stimulus, we used 3.1 version Facegen Modeller program (*http://facegen.com*) to generate emotional face pictures with oriental characteristics, and in order to have control over the interference effect of such background information as hair and neck on face identification, we tailored the face stimulus into oval shape. Emotion identification was carried out by 15 postgraduates majored in psychology over 20 groups of face pictures, selecting pictures with an emotional identification rate above 67% as target pictures for further screening, that is, at least 10 evaluators can identify the emotions accurately. And then we invited 30 undergraduates to appraise the arousal and valence of preliminarily screened emotional faces on the scale of 1-7. The grouping criteria for happy faces is valence scoring over 5, and arousal scoring over 5; the grouping criteria for angry faces is valence scoring less than 2, and arousal scoring over 3; the grouping criteria for neutral faces is valence scoring between 3 and 5, and arousal scoring less than 3 (**DAI Qin & FENG Zhengzhi, 2009**). Finally, we selected four groups of faces (including two groups of male faces and two groups of female faces), each group comprised of happy, angry and neutral faces of the same person. A face search set consists of four face pictures of a person against black background, with the point of fixation "+" appearing in the center of the field of vision of the subjects. Face pictures are presented in a diagonal position with the point of fixation in the center as the center, at 8.5° angle of view from the position of the subject.

2.3 Experimental Procedures

Visual stimulus is displayed on 14-inch notebook screens. The subject sits down 0.7m away from the notebook screen in a closed room. The experiment firstly displays point of fixation 500-1000ms, and then immediately the face search set 500ms, and requires the subject to judge whether the emotional valence of the four face pictures are consistent, by pressing "F" for consistency, and "J" for inconsistency. The subject has 1800ms to respond until the end of pressing, requiring the subject to make pressing response as quickly and accurately as possible. The time of interval between trials is 500-1500ms, during which only black background is displayed. The flow chart of the experiment is as shown in Table 1, consisting of five blocks, with each block including 128 trials, and 2-3 min of rest between blocks.

Prior to the commencement of the task, instructions are presented and explained to the subject, followed by 10 trials of experiment exercise, enabling the subject to understand the experimental task. During the experiment, the subject is required to continually stare at the point of fixation at the center of the screen, control the blinks to occur during the intervals between trials, in order to avoid excessive eye movement. The whole experiment continues for a period of 1.5 hours.

3 Results

At the time of analyzing the behavioral data, we deleted trials of wrong response by the subjects, and rejected data of response outside of the two standard deviations, and the final valid data is not less than 95%. In previous researches, the rate of accuracy is generally not taken as the object of examination, therefore, in this study, the reaction time is the only dependent variable.

This study compares the reaction

time of two groups of subjects under three conditions, i.e. interference stimulus by neutral face, and target stimulus by angry and happy faces (see Table 1). We conducted repetitive measurement deviation analysis by the following formula: 2 (groups: violent group vs non-violent group) × 3 (target stimulus types: happy face vs angry face), based on different target stimulus. The group is the factor between the subjects, and target stimulus type is factor of the subjects. The results show that, the main effect of both the group ($F_{(1.21)}$=7.997, P=.010) and the target stimulus ($F_{(2.42)}$=14.573, P=.000) are remarkable, and so is the interactive effect ($F_{(2.42)}$=3.492, P=.040). The result of simple effect analysis of fixed target stimulus types shows that, when the target stimulus is angry face, the reaction time of the violent group is faster than that of the non-violent group, and the difference is notable ($F_{(1.21)}$=11.555, P=.003). when the target stimulus is happy face, the reaction time of the violent group is also faster than that of the non-violent group, and the difference is also notable ($F_{(1.21)}$=7.841, P=.011). The result of simple effect analysis of fixed groups shows that, when the target stimulus is angry face, the reaction time of the violent group is remarkably shorter than that of happy face and neutral face (P=.000; P=.003), nevertheless, the reaction time of the non-violent group toward angry, happy and neutral faces has no notable difference (P=.098; P=.738).

In a summary of the above experimental results, with respect to positive and neutral emotional clues, the violent group has notable attentional bias toward negative emotional clue, and the non-violent group does not show remarkable attentional bias. In comparison with the non-violent group, the violent group shows more sensitive characteristics toward negative emotional stimulus.

4 Discussion

As shown by the results, reaction of robbery offenders toward angry face in neutral face set is faster than that of happy face in neutral face set and all neutral faces, and the difference is remarkable (P=.000; P=.003), and the control group has no notable difference toward the three valence of facial expressions (P=.098; P=.738), and the reaction of robbery offenders toward angry face is obviously faster than that of the control group ($F_{(1.21)}$=11.555, P=.003). This verifies our assumption from the angle of behavior that, in comparison with positive stimulus (happy face), robbery offenders have attentional bias toward negative stimulus (angry face), and are more sensitive toward negative emotion than the control group. Here the results of research on AES by **Hansen and Hansen** (**1988**), and **Purcell** (**1996**) are reproduced, i.e. the reaction toward angry face is obviously faster than happy face. This asymmetry in processing different valence of emotional faces suggests that, when perceiving emotional faces, the cognitive system considers that faces containing threat information (angry faces) are more important than emotional faces conveying good faith and friendliness (happy faces), and more meaningful to the existence of the mankind (e.g., **Treisman & Souther, 1985**), therefore, negative stimulus with potential dangerous factors can acquire attention in priority.

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Table 1. Reaction Time of Judgment against the Background of Angry, Happy and Neutral Faces (M±SD), Unit: ms.

Group	Target stimulus		
	Angry face	Happy face	Neutral face
Violent group (n=12)	526.03+33.86	551.93+31.97	559.40+30.23
Non-violent (n=11)	620.72+89.95	628.36+88.64	624.25+100.99

The Study on the Impact of ATM Gene Silencing on Hela Cell Radiosusceptibility

LUO Judong [1, 2, #], GE Yangyang [2, #], ZHOU Xifa *[, 1], LU Xujing [1], ZHANG Shuyu [2], TANG Hua [1], LING Yang [1], CHEN Ling [1], CAO Jianping *[, 2]

[1] Changzhou Tumor Hospital, Soochow University, Changzhou, 213001, China
[2] School of Radiation Medicine and Protection; Jiangsu Provincial Key Laboratory of Radiation Medicine and Protection, Soochow University, Suzhou 215123, China

ABSTRACT

Objective By using conventional chromosome aberration analysis, the radiosensitivity of Hela cells by silencing ATM gene (Hela^{ATM-} cell) using siRNA technology was investigated.

Methods (1) ATM siRNAs were designed and synthesized, including four pairs of siRNAs specifically targeting ATM gene, a negative control siRNA and a FAM-marked siRNA as a negative control. Transfections of these siRNAs into Hela cells were performed using liposome, transfection efficiency was monitored under fluorescent microscope. ATM expression of transfected Hela cells was detected at different time by using RT-PCR assays. (2) By using conventional chromosome aberration analysis method, chromosome aberration frequencies (CAF) of Hela^{ATM-} cells and Hela cells exposed to ^{60}Co γ-ray were observed compared to control Hela cells.

Results (1) FAM-marked negative control siRNA was successfully transfected into Hela cells as monitored by fluorescent microscope. (2) The data with RT-PCR assays showed that ATM gene expression was significantly depressed in Hela^{1057} cells (transfection with 1057-1075nt siRNA) 24h following transfection ($p<0.05$). The 1057-1075nt siRNA worked until 96h after transfected ($p<0.05$). ATM gene expression was decreased significantly at 24h and 48h after transfected compared to 96h ($p<0.05$), no significant difference between 24h and 48h, 72h and 96h after transfected ($p>0.05$); ATM gene expression was decreased significantly at 24h and 48h than 72h and 96h after transfection ($p<0.05$). (3) After exposed to 0, 1, 2, 3, 4 and 5 Gy ^{60}Co γ-ray, the main pattern of chromosome aberration was dic and the radiation-induced level of CAF was significantly higher in Hela^{ATM-} cells than in control Hela cells at each dose point ($p<0.01$). In the two cells, CAF had a positive correlation with the doses used, and their linear regression equation was $Y=a+bD$. The slope of CAF linear regression equations of Hela^{ATM-} cells was larger than that of control Hela cells ($p<0.05$).

Conclusion (1) ATM gene was successfully silenced by transfection of chemically synthesized siRNAs in Hela cells, named Hela^{ATM-}. (2) The CAF of Hela^{ATM-} cells was significantly higher than control Hela cells by using conventional chromosome aberration analysis.

Key Words

RNAi, ATM, Radiosensitivity, Conventional chromosome aberration analysis method.

Correspondence to:

ZHOU Xifa, CAO Jianping

E-mail: lhpljd@yahoo.com.cn

The first two authors[#] contributed equally to this work.

The gene mapping of ATM (ataxia telangiectasia mutated, ATM) is located on human chromosome 11q22-23, and its total length is about 150Kb, with a coded sequence around 12Kb. 3056 amino acids consists of the protein, and the relative molecular weight is about 350 KDa. ATM contains the Wortmannin-inhibited Ser/Thr protein kinase active site, thus belonging to the PI-3K (phosphatidylinositol 3-kinase) kinase family [1]. When factors such as ionizing radiation generate DNA damages, the dominant kinase ATM can be initiated and directly feel DNA double-stranded breaking (DSB) damage signals, which can catalyze the phosphorylation of a variety of important functional substrate proteins, participate in cell cycle regulation, DNA damage signal transduction and the repair of DNA damage, and maintaining the chromosomal stability. Thus, ATM is the hub of the cell response signal transduction pathway [2]. However, whether ATM expression is associated with chromosome aberration is still unknown.

RNA interference (RNAi) indicates that the introduction of endogenic and ectogenic double strand RNA (double-stranded RNA, dsRNA) into cells of organisms, which generate the the degradation of homologous mRNA specificity, and the later post-transcripitional gene silencing (PTGS) [3,4]. For small interfering RNA (siRNA) with a length of 21-23nt, they are key intermediate effector molecules of RNAi. siRNA applies sequence specificity to inhibit target gene expressions, and its high efficiency and high specificity

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demonstrate outstanding advantages in the study of gene function and gene therapy [5,6].

According to the design principles of Tuschl T siRNA [7], siRNA on ATM gene was designed and synthetized to transfect tumor cells and observe the gene silencing effects. This can provide models for the research into biological characteristics of the tumor cells with ATM afunction. The conventional chromosome aberration analysis method was adopted to study on its radiosusceptibility, and provided theoretical and experimental basis for further research on how to improve tumor radiosusceptibility.

1 Materials and Methods

1.1 Materials

Hela cervical cancer cells (Shanghai Cell Bank, Chinese Academy of Sciences); DMEM medium and newborn calf serum (Invitrogen); RNeasy Mini Kit (QIAGEN); QIAshredder Homogenizers (QIAGEN); Lipofectamine^{TM}2000 (Invitrogen); One Step RT-PCR Kit (TaKaRa); 4 pairs of siRNA, positive and negative control siRNA (Shanghai Jima Company); PCR primers: ATM gene primer, GAPDH primers , β-actin primers (Shanghai Sangon); colchicine, Giemsa (Sigma).

1.2 Methods

1.2.1 Design and Synthesis of siRNA Sequences

According to the design principle of siRNA, four pairs of siRNA were designed and synthesized for ATM mRNA (NM_000051), as well as one pair of negative control siRNA NC, one pair of positive control siRNA PC (for GAPDH mRNA and NM_002046). In addition, one pair of negative control FAM-NC of marked FAM was synthesized for the observation of the observed effects under a fluorescent microscope. All siRNAs were synthesized by Shanghai Jima Company. Each sequence is shown in Table 1.

1.2.2 Cells Transfection

Hela cells were seeded in DMEM medium supplemented with 10% fetal bovine serum for serial subcultivation in the incubator with 5% CO_2 at 37°C, and the passage number <20. 24h before transfection, (1-3)×10^5 Hela cells were seeded in 6-pore plates, so that the cell density in the transfection can reach 30-70%. In the transfection, the serum-free DMEM medium was replaced. According to 5ul Lipofectamine^{TM}2000 per pore: 150pmol siRNA was used to transfect Hela cells (refer to the operations on the manual). 5h after the transfection, DMEM medium supplemented with 10% fetal bovine serum was replaced. The above experiments were divided into 5 groups: A. 4 pairs of transfected ATM siRNA; B. positive control siRNA transfected PC group; C. negative control siRNA transfected NC group; D. normal Hela cells; E. FAM-NC negative control transfected group. Cells of each group were named after the transfected siRNA sequence, such as the transfected Hela cells of 1057-1075nt siRNA were named as Hela^{1057}, and cells with interference effects were named as Hela^{ATM-} cells.

1.2.3 the transfection efficiency of the cells of the group E observed under the fluorescence microscope

1.2.4 One Step RT-PCR

RNeasy Mini Kit was used to extract the total mRNA of cells in each group. OD values were measured with a nucleic acid UV spectrophotometer, with A260/A280 of 1.8~2.1 for ATM mRNA expression 24h after the A, C, D cells transfected siRNA. The primer design was completed with Primer Premier5.0, and synthesized with GenBank Blast analysis. β-actin mRNA (NM_001101) was selected as the internal standard to identify the relative expression mRNA level of ATM gene.

(1) Sequences are as follows:

ATM Primer Sequence:

Forward Primer: 5'-GCATTACGGGTGTTGAAGGTGTC-3'
Reverse Primer: 3'-AGAAACTGACCTGGTACTTAGGAA-5'

β-actin Primer Sequence :

Forward Primer: 5'-ACGACCCATTCGAACGTCTG-3'
Reverse Primer: 3'-CTCCCTCGGACTCTTTGCC-5'

(2) The reverse-transcription reaction system:

Hela mRNA	A or B or C or D Group'	0.1ug
ATM or β-actin or GAPDH	Forward Primer (20uM)	0.2ul
	Reverse Primer (20uM)	0.2ul
2×One Step RT-PCR Buffer		12.5ul
TaKaRa EX Taq HS (5U/ul)		0.5ul
M-MLV RTase(Rnase H free,200U/ul)		0.25ul
RNase Inhibitor(40U/ul)		0.5ul
RNase Free dH_2O	Supplmentary 25ul system	

(3) The PCR conditions:

ATM: RT: 42°C 15min, 95°C 2min, PCR: 95°C 5s , 60°C 20s , 72°C 15s , 28 cycles.

β-actin: RT: 42°C 15min, 95°C 2min , PCR: 95°C 5s , 56°C 20s , 72°C 15s , 28 cycles.

Table 1. Designed siRNA sequences

No.	Corresponding Location on mRNA	Nucleotide Sequence	GC Content (%)
1	1057-1075nt	Sense 5'-GAGCUCUUCAGGUCUAAAUdTdT-3' Antisense 5'-AUUUAGACCUGAAGAGCUCdTdT-3'	39.0
2	4661-4679nt	Sense 5'-GCAGCUGAAACAAAUAAUGdTdT-3' Antisense 5'-CAUUAUUUGUUUCAGCUGCdTdT-3'	33.3
3	5746-5764nt	Sense 5'-AGGCCUGGAUGAUAUAAAUdTdT-3' Antisense 5'-AUUUAUAUCAUCCAGGCCUdTdT-3'	33.3
4	7192-7210nt	Sense 5'-GCUCCCUGAAAGGGCAAUAdTdT-3' Antisense 5'-UAUUGCCCUUUCAGGGAGCdTdT-3'	47.6
5	negative control NC	Sense 5'-UUCUCCGAACGUGUCACGUTT-3' Antisense 5'-ACGUGACACGUUCGGAGAATT-3'	47.6
6	positive control PC	Antisense 5'-AUUUAGACCUGAAGAGCUCdTdT-3' Antisense 5'-CUUGAGGCUGUUGUCAUACTT-3'	42.8

(4) The product analysis:

The PCR products were treated with 1.5% agarose gel electrophoresis, photoed with the ultraviolet image analyzer and semi-quantitative analysis with Bandscan5.0 Gel Image Analysis Software. The relative expression level of Hela cell ATM mRNA =ATM cDNA/ β-actin cDNA. ATM amplification product is 138bp and β-actin amplification product is 564bp.

1.2.5 Ionizing Radiation

At room temperature, ^{60}Coγ ray was used to irradiate Hela cells and Hela^{ATM-} cell (24h after the transfection of siRNA) in the logarithmic phase, and the adsorbed dose is 0, 1, 2, 3, 4, and 5Gy (dosage rate as 1.0Gy/min). After the ^{60}Coγ ray irradiation, the medium was repalced immediately and added with colchicine (with the final concentration as 0.10μg/ml), and the medium was cultured for another 24h.

1.2.6 The Preparation of Chromosome Specimens

0.25% trypsin was used to harvest cells, which were then removed in a centrifuge tube, treated with centrifugation at 1,500r/min for 8min. The upper layer of medium was removed and cells were harvested with centrifugation. The cells were treated with low permeability of 0.075M KCl and fixed with fresh fixative (methanol: ice acetic acid = 3:1), conventional droplet sheet and Giemsa staining, eventually producing the chromosome fragments.

1.2.7 Chromosome Aberration Analysis

Metaphase with sound chromosomes dispersity was selected under the microscope, and various chromosomal aberration types were counted, mainly including dicentric mitochondrial (dic) and centromere ring (r). The blind reading was adopted so that the distortion seen by an observer must be reviewed by another observer.

1.2.8 Statistical Analysis

The statistical software of SPSS13.0 for windows was adopted. The RT-PCR experimental data was expressed by mean ± standard deviation. The relationship between chromosome aberration rate and dose of Hela cells and Hela$^{ATM—}$ cell was treated with linear correlation analysis. Meanwhile, the distortion rate of each dose point between two cells was compared with One-Way ANOVA.

2 Result

2.1 Observation of the efficiency of the cells transfected with the siRNA

The transfection efficiency of cells after the transfection of siRNA was observed under an inverted fluorescence microscope. A, B are photos under an ordinary microscope, and C is the photo under a fluorescence microscope. With the comparison of three groups, it can be observed that in A, there is no bright spot in Hela cells with high refractive index; in B, there are bright spots in Hela^{1057} with high refractive index, as the mixation of Lipofectamine^{TM}2000 and siRNA; in C, the green fluorescent in cells is simulated and initiated by FAM, consistent with bright spots in cells in B, indicating that Lipofectamine^{TM}2000 successfully mediated siRNA- transfected Hela cells (as shown in Figure 1).

2.2 Interference Effect Screening of siRNA Inhibition of ATM Gene

At 24h of the siRNA transfection of Hela cells, β-actin and ATM mRNA expressions are shown in Figure 2A. The relative expression quantity of ATM mRNA in cells is shown in Table 2 and Figure 2B. At 24h, 48h, 72h, 96h of 1057-1075nt siRNA transfection of Hela^{1057} cells, RT-PCR results of ATM and β-actin are shown in Figure 3A, and the relative expression of ATM mRNA is shown in Figure 3B.

As shown in Figure 2A, cells present

Table 2. Relative expression of ATM mRNA

No.	Cells	ATM/β-actin
1	Hela	0.76±0.0764
2	Hela^{NC}	0.65±0.0289$^{\#}$
3	Hela^{5746}	0.58±0.1670
4	Hela^{7192}	0.59±0.0702
5	Hela^{4661}	0.45±0.1222
6	Hela^{1057}	0.30±0.1155*

** P<0.05 (Hela^{1057} vs Hela);*
$^{\#}$ P>0.05 (Hela^{NC} vs Hela); n=3.

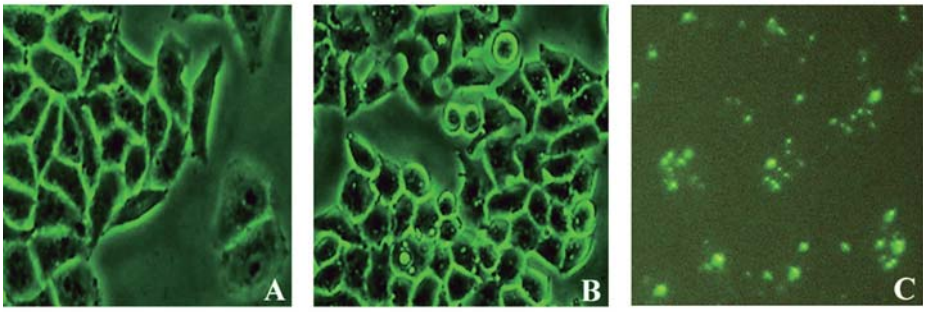

Fig.1 Photos of cells from each group under a fluorescence microscope. A. Hela, B. Hela^{1057}, C. HelaFAM-NC; bright spots in Hela^{1057} with high refractive index, as the mixation of Lipofectamine^{TM}2000 and siRN; C, green fluorescence in cells, as siRNA marked with FAM. Magnification: 10×20.

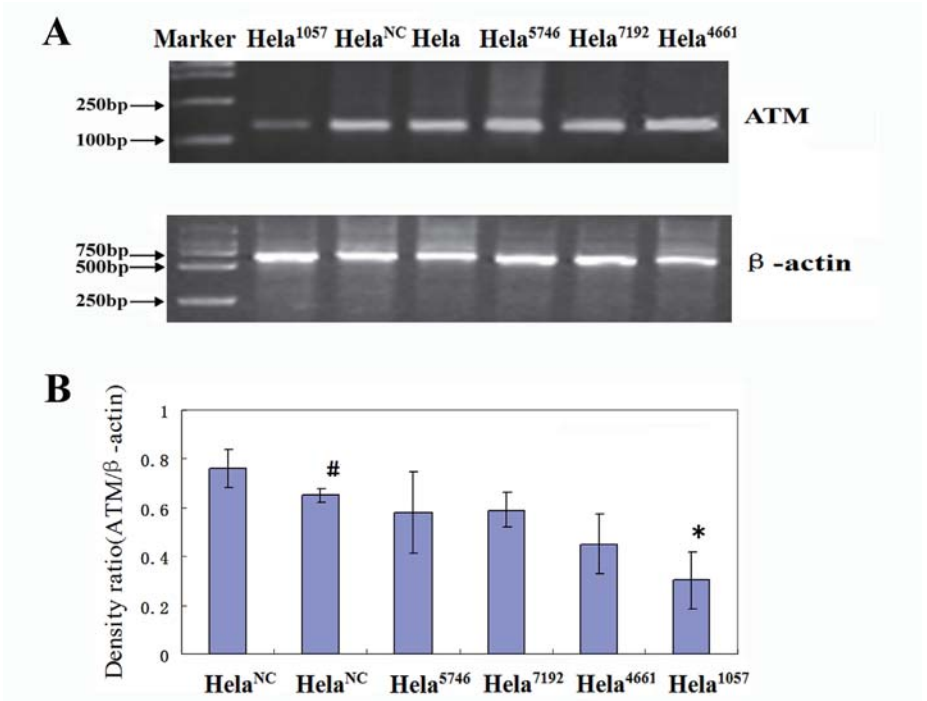

Fig.2 (A) RT-PCR analysis of ATM and β-actin expression at the 24h after the transfection. ATM PCR product 138bp and β-actin PCR product 564bp. (B) Relative Expression of ATM mRNA at 24h after the transfection. Relative expression of ATM cDNA/β-actin cDNA, where *P<0.05 (Hela^{1057} vs Hela), #P>0.05 (Hela^{NC} vs Hela), n=3.

a β-actin target band at 564bp with luminance uniformity, and ATM target band at 138bp with vairous luminance, among which Hela^{1057} is the weakest. As shown in Table 2 and Figure 2B, as for the relative expression of ATM mRNA, Hela cells are the highest, and the relative expression of ATM mRNA of siRNA-transfected Hela^{5746}, Hela^{7192} , Hela^{4661}, Hela^{1057} was reduced to different degrees. Among them, ATM mRNA relative expression of Hela^{1057} was lower than Hela cells (P<0.05), while that od Hela^{5746}, Hela^{7192} and Hela^{4661} did not present significant difference than Hela cells (P>0.05), indicating that 1057-1075nt siRNA has a more intensive interference effect on Hela ATM gene expression. Other siRNA sequences did not present obvios interference effects.

The ATM mRNA relative expression level of Hela^{NC} in the negative control did not present significant difference from Hela (P>0.05), demonstrating that negative control nonspecific nucleic acid sequence can not inhibit the expression of the ATM gene in Hela cells and impose no interference effects. At the same time point, compared with the relative expression of Hela^{1057} and Hela ATM mRNA, significant difference can be observed (P<0.05). Compared with Hela^{NC} and Hela cells, no significant difference can be observed (P>0.05), indicating that 1057-1075nt siRNA can effectively interfere with the ATM gene, and the duration extends to 96h after the transfection with siRNA. As shown in Figure 3A and 3B, in the comparison of Hela^{1057}ATM mRNA at 4 time points, 24h, 48h and 96h after the siRNA transfection present significant difference (P<0.05). Comapred with 96h after the transfection, 72h after the siRNA transfection showed no significant difference (P>0.05), indicating that 1057-1075nt siRNA interfering ATM gene has the optimal effect at 24h and 48h of the transfection, while 72h and 96h showed no significant difference (P>0.05).

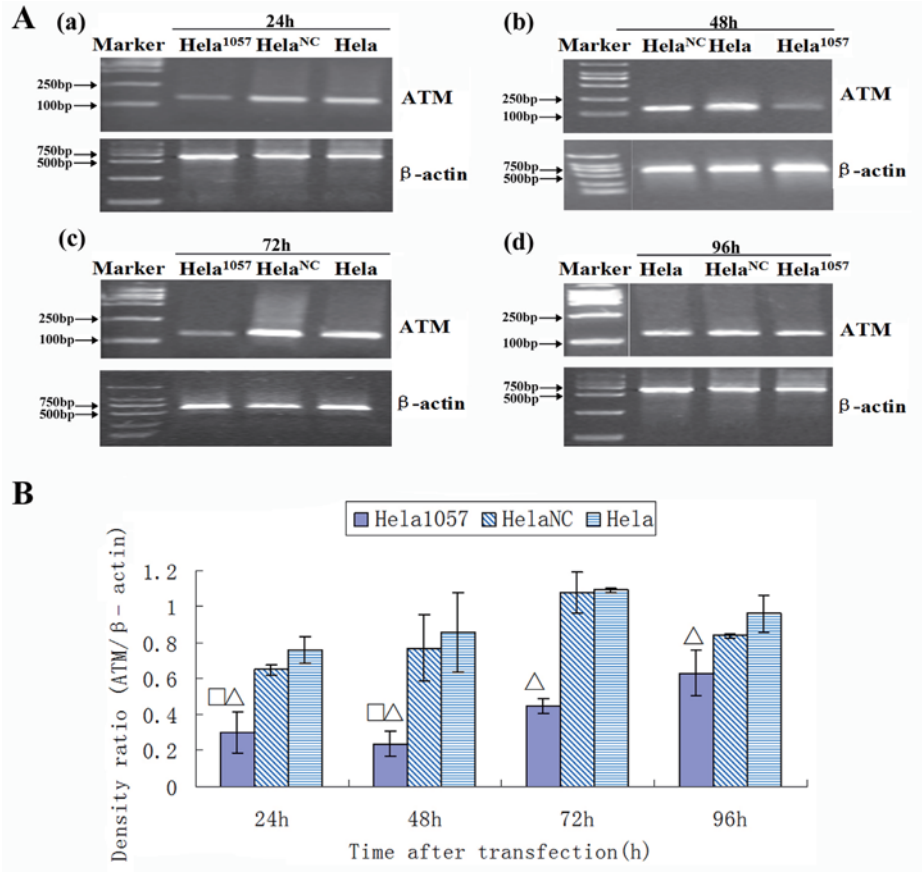

Fig.3 (A) ATM and β-actin RT-PCR electrophoretogram at different time points. (a), (b), (c) and (d) are ATM and β-actin RT-PCR electrophoretogram at 24h, 48h, 72h and 96h after the siRNA transfection. (B) Relative expression of ATM mRNA at different time points. At different time points of 1057-1075nt siRNA transfection of Hela ells, ATM/β-actin relative expression, where ΔP<0.05 (Hela^{1057} vs Hela) , □P<0.05 (Hela^{1057} cell, 24h vs 96h, 48h vs 96h) , n=3.

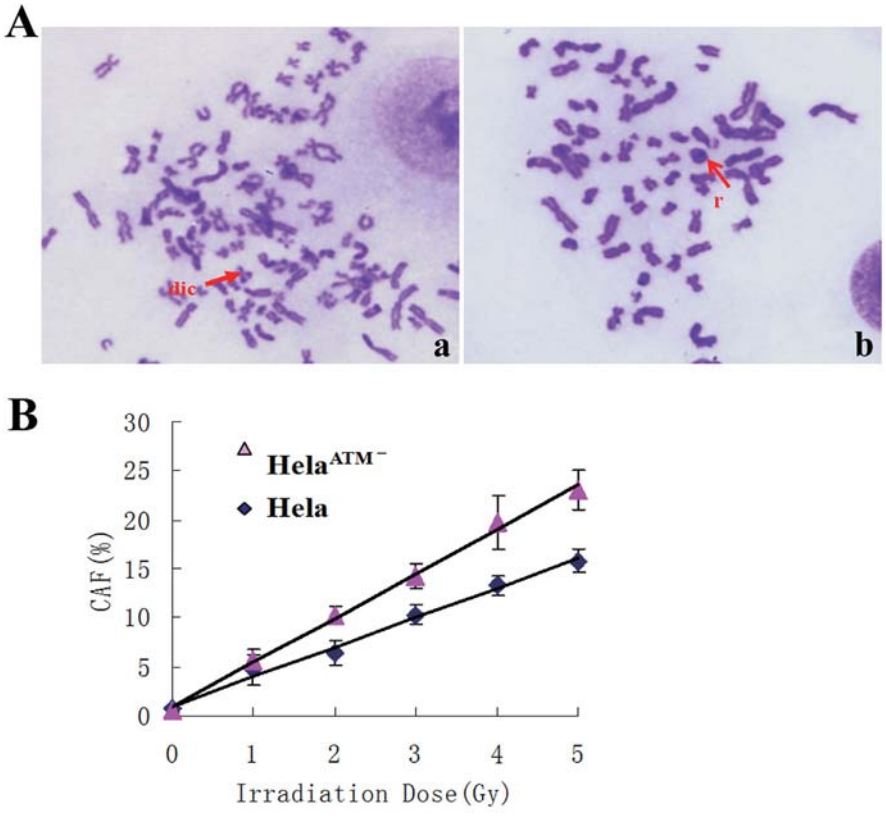

Fig.4 (A) ^{60}Coγ ray-induced Hela and Hela^{ATM-} CA. (a) presents dic, (b) presents r, and the magnification times: 10×100. (B) The relationship between Hela/Hela^{ATM-} CA Rate and the exposure dose (Gy).

2.3 Hela and Hela^{ATM-}Cell Chromosome Aberration Induced by ^{60}Coγ Ray Radiation

In the experimental irradiation dose range, the main type of chromosomal aberrations observed is the dicentric mitochondria. Figure 4A presents the dicentric mitochondria and centromere ring observed under the microscope.

Results of Hela and Hela$^{ATM—}$ cell chromosome aberration induced by ^{60}Coγ ray radiation are shown in Table 3. It can be seen in Table 3 that after the irradiation of 0, 1, 2, 3, 4, and 5Gy ^{60}Coγ ray, the Hela and Hela$^{ATM—}$ cell chromosome aberration increases with rising dosage (P<0.01); except 0Gy, at the same dosage point, aberration rate of Hela$^{ATM—}$ is obviously higher than Hela cells, where 3Gy point showed significant difference (P<0.05), and the difference of the rest dosage showed extreme significance (P<0.01), indicating that after the irradiation, the degree of injury of HelaATM— chromosome cells was more severe than Hela cells and the radiosensitivity of Hela$^{ATM—}$ cells is higher than Hela cells.

2.4 The Relationship between Hela and Hela$^{ATM—}$ CA Rate and the Exposure Dose

Observation results from Table 3 and the correlation analysis showed that Hela and Hela$^{ATM—}$ CA rate is positively correlated with the exposure dose; thus the relationship is shown in Figure 4B and the linear regression equation and the fitting equation are as follows:

Hela$^{ATM—}$ cell chromosome aberration rate: $Y=0.89+4.53D$, $R^2=0.97$, r= 0.98 (P<0.01);

Hela cell chromosome aberration: $Y=1.00+3.01D$, $R^2=0.98$, r= 0.99 (P<0.01).

Where Y is the chromosome aberration rate (%), D is the exposure dose (Gy). It can be seen from the equations that the slope of the linear regression equation of Hela$^{ATM—}$ cell CA rate is significantly greater than Hela cells, presenting significant difference (P<0.05). This indicates that compared with Hela cells, Hela$^{ATM—}$ CA rate grows faster than that of Hela cells along the increase of the dosage.

3 Discussion

ATM gene mutations lead to ataxia-telangiectasia (AT)[8-10], which is a rare autosomal invisible cancer tendency hereditary disease involving the nerves, blood vessels, skin, reticuloendothelial system and endocrine system, clinically presented as progressive cerebellar degeneration, high radiosusceptibility, immunodeficiency, precocious and tumors with high incidence. However, whether ATM expression is associated with chromosome aberration is still not clear.

RNAi extensively exists in advanced plants and animals, as a gene silencing phenomenon mediated by siRNA. RNAi, as an efficient new technology for the specific inhibition of gene expression, has been extensively used in the study of gene function and gene therapy. Theoretically, any genes of human cells can be silenced by RNAi. Commonly used methods to obtain siRNA include chemical synthesis, in vitro transcription, degradation of long fragment of dsRNAs through RNase III, in vitro preparation of siRNA, and cell expression with PCR-prepared siRNA expression cassettes, siRNA expression vector or viral vectors. Each method has advantages and disadvantages, and its application shall take into account the siRNA preparation, difficulty of transfection, the observation of the transfection efficiency, and effect maintaining duration and so on.

Currently, one of the focuses of cancer research is the seeking for sensitizers with high efficiency and low toxicity, that is, to improve the tumor radiotherapy and chemotherapy sensitivity. Among populations, ATM heterozygous carriers account for about 1-2%, their tumor incidence was significantly increased [13]. Vairous studies verified that ATM gene can be used as the potential target for cancer gene therapy [14,15]. In cancer gene therapy, to introduce artificially synthesized siRNA or contructing the siRNA expression vector in tumor cells can specifically inhibit the target gene expression.

In this study, with the chemical synthesis of sequence-specific siRNA for ATM, liposomes were transfected into Hela cells to establish the missing tumor cell model of ATM, thus providing basis for for further study of the tumor radiotherapy and chemotherapy sensitivity. RT-PCR results presented that chemically synthesized siRNA transfected and mediated by liposome entered the Hela cells and successfully inhibited the expression of ATM mRNA (P <0.05). Its interference effects can be continued until 96h after transfection with siRNA, and nonspecific siRNA imposes no impact on ATM mRNA experssion (P<0.05).

The transfection efficiency is an important factor of the experiment, and the transfection method, target gene and cell selection shall be considered. This experiment optimized transfection conditions, and adopted 5ul Lipofectamine^{TM}2000 per pore: 150pmol siRNA transfection of Hela cells (50-60%) presents satisfying effects. In this study, as the transient transfection method is adopted, the long-term maintenance of interference effect of target gene needs to be further explored[16,17]. The application of siRNA in ATM-missing model of other tumor cells, especially radiation-resistant tumor cells (such as glioma cells) should be further studied.

Radiation-induced chromosomal aberrations (CA) make daughter cells after the split unable to obtain a complete set of chromosomes, and die for failing to continue the further splitting. CA is the change of chromosome morphology or structure, and is also a sound index of ionizing radiation damage of cell population, which not only reveals the ionizing radiation damage and evaluates the extent of damage, but also can predict the exposure dosage of personnel under accidents with the established CA dosage-efficiency curve established by the irradiation on in vitro human peripheral blood. Conventional chromosome aberration analysis technique is simple and convenient, thus is used in this study to measure the radiosensitivity of Hela$^{ATM—}$ cells. Various types of

Table 3. CA rate comparison of Hela and Hela$^{ATM—}$ cells after the ^{60}COγ irradiation

dose point Gy	Analysis cell amount	Hela Cells			Hela$^{ATM—}$ Cells		
		dic	r	Total aberration rate (%)	dic	r	Total aberration rate (%)
0	600	4	0	0.67±0.19	4	0	0.67±0.13
1	400	19	0	4.75±1.62**	23	0	5.6±1.15**##
2	200	13	0	6.50±1.35**	19	1	10.0±0.87**##
3	200	20	1	10.5±1.01**	27	1	14.0±1.23**#
4	200	26	0	13.0±2.75**	40	0	20.0±2.75**##
5	200	31	1	16.0±1.21**	45	1	23.0±2.02**##

*** P<0.01, comparison of Hela and Hela$^{ATM—}$ cells at 1, 2, 3, 4, 5Gy with 0Gy;*
 # P<0.05, ## P<0.01, comparison of Hela and Hela$^{ATM—}$ at the same dose point, n=3.

chromosomal aberrations are observed, including the chromosomal pattern aberrations, chromatid aberrations and chromosome amount distortion. The biological dosimetry adopted centromere free ring (r0), dicentric chromosome (dic) and centromere ring (r) and other unstable chromosome aberration frequency to estimate doses. In this study, dic and r are used to calculate the distortion rate of the chromosome.

With conventional chromosome aberration analysis, it is found that after the irradiation with ^{0}Coγ at 0, 1, 2, 3, 4, and 5Gy, CA of Hela and Hela^{ATM-} cells increases with the rising dosage. In addition, at the same dosage point, CA rate of Hela^{ATM-} cells is higher than Hela cells, presenting significant difference ($P<0.01$). This indicates that at the same irradiation dose point, Hela^{ATM-} chromosome damage is greater than Hela cells. For the above materials, according to four kinds of mathematical models provided by WHO ($y=a+bD$, $y=a+bD+cD^2$, $y=kDn$, $y=a+cD^2$) and statistical methods of Wang Jixian and others, curve fitting, regression coefficients and the degree of fitting are tested. $Y=a+bD$ mode is selected as the optimal regression equation. The fitting equation is as follows:

Hela^{ATM-} cell chromosome aberration rate: $Y=0.89+4.53D$, $R^2=0.97$, $r= 0.98$ ($P<0.01$);

Hela cell chromosome aberration: $Y=1.00+3.01D$, $R^2=0.98$, $r= 0.99$ ($P<0.01$).

CA rate of Hela^{ATM-} and Hela cells is positively related with the irradiation dose, and the slope of the linear regression equation of Hela^{ATM-} cell CA rate is significantly greater than Hela cells, presenting significant difference ($P<0.05$). This indicates that compared with Hela cells, Hela^{ATM-} CA rate grows faster than that of Hela cells along the increase of the dosage. The larger the dosage is, the more severe the Hela^{ATM-} chromosome damage is.

After the ionizing radiation of cells, the radiation signals were transduced by the reactive oxygen species (ROS) and DNA strand breaks, which induce the phosphorylation chain reaction of intracellular some columns molecules. Through induction of cell cycle G_1, G_2, and S delay as well as S/M uncoupling mitosis delay, the ionizing radiation influences the cell cycle progression. ATM gene coding protein was involved in cell cycle control, DNA damage signal transduction and repair, stability maintaining and other processes. ATM gene silencing leads to the loss of ATM kinase activity. Ionizing radiation signal failed to activate ATM kinase, thus failing to induce p53 phosphorylation so that p53 regulation was weakened or delayed, and the damaged DNA synthesis was inhibited. This hinders the process of DNA repair, and generates chromosomal instability so that cells became extraordinarily sensitive to the damage factors such as ray, performing features such as enhanced radiosensitivity. In this way, spontaneous CA rate and radiation-induced CA rate became higher than normal cells. These are demonstrated in AT cells with ATM gene mutations [18].

Hela cells are cervical cancer cells transformed by Human Papillomavirus 18 (HPV18). Generally, cervical cancer cell lines contain high gene expressions of HPV E6/E7. The integration of E6 protein, p53 protein, and intracellular E6-associated protein (E6-AP) can promote p53 degradation and its inactivation, so that cells enter S stage from G_1. E7 protein was then integrated with Rb tumor suppressor gene, affecting the integration of Rb and E2F, so that E2F was separated from Rb, entering the gene transcription at S stage. As Hela cells lack G_1 delay, spontaneous CA rate and radiation-induced CA rate became higher. Interfered ATM gene of Hela^{ATM-} cells leads to ATM afunction, also presenting features similar to high radiosensitivity of AT cells, and its radiosensitivity is higher than Hela cells.

In summary, we found that ATM gene was successfully silenced by transfection of chemically synthesized siRNAs in Hela cells, named Hela^{ATM-}. The CAF of Hela^{ATM-} cells was significantly higher than control Hela cells by using conventional chromosome aberration analysis.

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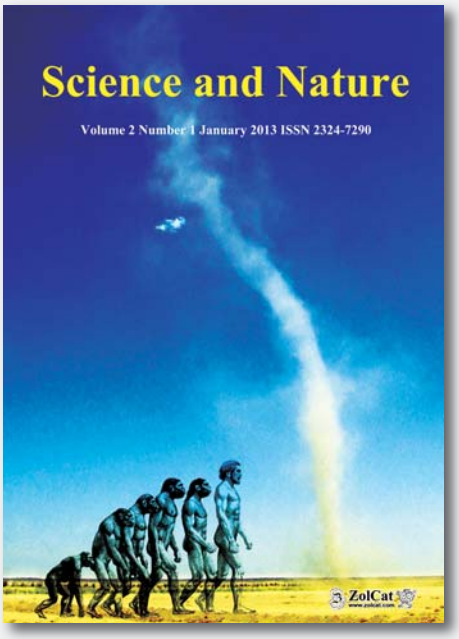

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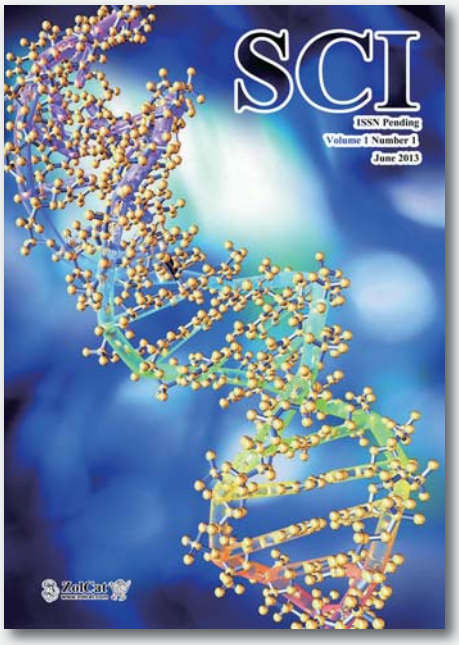

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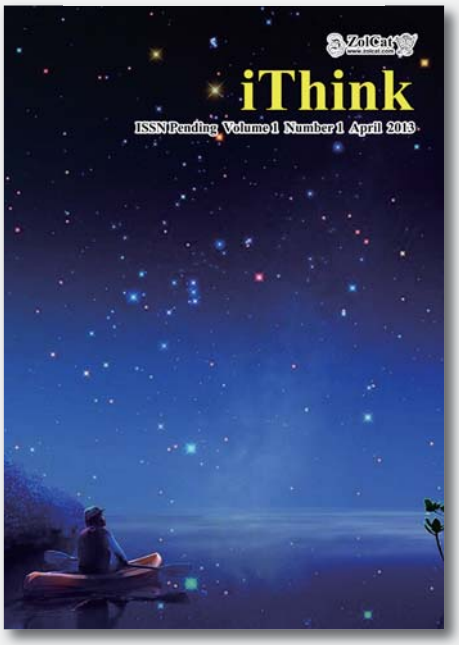

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