Research Article

Detection of TP53 R249 Mutation in Iranian Patients with Pancreatic Cancer

Ashraf Mohamadkhani,1 Elnaz Naderi,1 Maryam Sharafkhah,1 Hamid Reza Fazli,2 Malihe Moradzadeh,3 and Akram Pourshams1

1 Liver and Pancreatobiliary Diseases Research Center, Digestive Diseases Research Institute, Tehran University of Medical Sciences, Tehran, Iran
2 Young Researchers Club, Ahar Branch, Islamic Azad University, Ahar, Iran
3 Faculty of Medicine, Mashhad University of Medical Science, Mashhad, Iran

Correspondence should be addressed to Akram Pourshams; akrampourshams@gmail.com

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The TP53 gene encodes tumor protein p53 which play a major role in the etiology of pancreatic cancer. The important role of codon 249 of TP53 for binding of p53 to its sequence-specific consensus site in DNA has been revealed by crystallography’s studies, and mutation at this codon was detected in the plasma of some human cancers. The TP53 Mut assessor software within the International Agency for Research on Cancer (IARC) TP53 Database was performed to evaluate every possible mutation at codon 249. DNA was extracted from the plasma of 133 pancreatic cancer patients and 85 noncancer-bearing individuals. Exon 7 in TP53 was amplified, and mutation at R249 was identified by the endonuclease cleavage of HaeIII. The group of patients showed a frequency of 11% (22 of 133 samples) R249 mutation compared to 3.5% (3 of 85 samples) in the group of control which was significant (P = 0.03). This mutation demonstrated statistically significant association with pancreatic cancer risk in unadjusted odds ratio (OR: 3.74, 95% CI: 1.1–13.2; P = 0.041); however when adjusted for confounding factors, it was marginally significant because of lower control samples. These findings demonstrate that mutation at R249 of TP53 can be considered for increasing risk of pancreatic cancer that needs more research.

1. Introduction

Pancreatic ductal adenocarcinoma (PDAC), the most common cancer of the pancreas, is the fifth commonest cause of cancer-related mortality among industrialized countries [1]. There is not much data regarding incidence and mortality of pancreatic cancer in Middle East. However the cancer of pancreas is not in the top 10 of cancer-related deaths in Iran [2]. The aggressive nature of the PDAC is related to poor prognosis; therefore, most of the patients at the time of diagnosis are harboring unsuitable cancer that is extremely resistant to chemotherapy [3]. The incidence rate of pancreatic cancer has been stabilizing over the past two decades in many developed countries; however, this continues to increase in countries where the rate of pancreatic cancer was relatively low four decades ago, such as Japan [4]. In Iran, pancreatic cancer is diagnosed in advanced stage with no identified risk factors. Pancreatic cancer is primarily an environmental disease and attributed mostly to cigarette smoking [5]; however the pathogenesis of this cancer also involves various genetic alterations particularly genes that controlled the cell cycle [6, 7]. The TP53 (tumor protein p53) as a main tumor suppressor gene is mutated in most of the tumors [8, 9]. The p53 protein inhibits cancer formation through regulating several pathways involved in cellular functions for cell cycle arrest and apoptosis [10].

Mutations of the genome of TP53 were currently at 60% of sporadic and 33% of familial pancreatic adenocarcinomas [11]. Mutations in the “hotspot” codons 175, 245, 248, 249, 273, and 282 cause distortions that create internal cavities or surface crevices in the protein scaffold leading to conformational changes in DNA binding surface [12].
Oligonucleotide array experiments showed that the TP53 link to the promoter regions and regulate approximately of 300 different genes through its DNA binding domain [13]. Moreover, the important role of codon 249 of TP53 for the function of this protein to bind to its sequence-specific consensus site in DNA has been studied by X-ray crystallography [14, 15]. Human cancer can be induced by environmental factors. For example, a substitution of serine for arginine in codon 249 of TP53, which is overcoming in areas with high exposure to aflatoxin B1, is associated with the incidence of hepatocellular carcinoma [16, 17].

Evaluation of mutation of TP53 might be used as a marker for incidence of cancers and may provide some clues about the significance of interaction of environmental and genetic factors. Several reports have offered that in some human cancers originated DNA from the tumor effectively can be extracted from the plasma or serum to study the specific missense mutation [14, 18]. Therefore, the aim of this study was to ascertain the prevalence of TP53 mutation at codon 249 in plasma in a case control study in patients with pancreatic ductal adenocarcinoma.

2. Patients and Methods

2.1. Patients. Blood samples were obtained from 133 pancreatic cancer patients and 85 noncancer-bearing individuals, and their plasma samples were stored at −70°C until use. Both case and control subjects were recruited between the years of 2010 and 2012. Cases were newly diagnosed patients attending at gastroenterology department of Shariati Hospital. The diagnosis of pancreatic cancer in patients was reconfirmed both histologically and clinically. Informed consent was obtained from all donors, and the protocol was approved by the institutional review board of the Shariati Hospital, Tehran University of Medical Science.

2.2. Evaluation of Mutations at Codon 249 of p53 Gene. All mutations of TP53 which was involved in human cancers are compiled in the International Agency for Research on Cancer (IARC) TP53 Database. The TP53 Mut Assessor software within IARC TP53 Database was used to evaluate the mutations at codon 249. This software as a novel stand-alone software allows the user to retrieve multiple information on every possible TP53 mutant whether or not they have been described in human cancer. According to the acquired results by this software, every mutation in codon 249 (R249S/G/I/K/M/N/T/W) is able to inactive the function of p53 protein that shows the importance of codon 249 in folding, structure and function of p53 protein.

2.3. Amplification of Exon 7 of TP53 and Mutation Detection. DNA was extracted from 200μL of plasma using a QiAamp blood kit (Qiagen) according to the manufacturer procedure. The standard PCR was performed in a total volume of 50μL as described previously [14] in which 50 ng template DNA, 10 pmol of each primer (sense: 5'-CTTGCCACAGGTCCTCCCAAA-3' and antisense: 5'-AGGGGTCAGCGGCAAGCAGA-3'), 0.25 mM of each dNTP, 2.5 mM MgCl₂, 5 units of HotStarTaq polymerase (Qiagen), and the appropriate volume of H₂O were used for amplification of a 236 bp region of exon 7 of the p53 gene in a “Touchdown” PCR program. The thermocycling conditions were 1 cycle of denaturation (5 min at 94°C), 20 cycles of denaturation (94°C for 45 s), annealing (63°C for 45 s, with −0.5°C per cycle), and extension (72°C for 1 min), followed by 30 cycles of denaturation (94°C for 45 s), annealing (60°C, 45 s), and extension (72°C for 1 min), and a final extension cycle of 72°C for 10 min.

The PCR products (exon 7) were digested with 10 units of the restriction endonuclease HaeIII (Fermentas, Hanover, MD), which cut within a GG|CC sequence encompassing codon 249 (AGG) in the wild-type sequence. However, mutation at codon 249 would lose the HaeIII restriction endonuclease site. Digestion of DNA was performed in a total volume of 20μL in which 5μL PCR products were added to 1μL HaeIII, 2μL 10 x buffer, and 12μL ddH₂O for 2-hour incubation at 37°C. The genotype screening was performed simultaneously for cases and controls. The detected mutations were validated by DNA sequencing analysis.

2.4. Statistical Analysis. All statistical analyses were performed with the Statistical Program for Social Sciences (SPSS, version 19 for Windows; SPSS Inc., Chicago, IL). The mean ± standard deviation (SD) was considered for continuous variables, and the frequency (%) was reported for categorical variables. Demographic variables were compared between two groups with independent t-test or pearson's chi-square test as appropriate. Simple and multivariable logistic regression was used to assess the effect of independent variables on TP53 mutation at codon 249 as a dependent variable. A P value of ≤0.05 denoted significance.

3. Results

3.1. PCR-RFLP Analysis of TP53 at Codon 249. To examine the TP53 mutation of codon 249 in patients with pancreatic cancer and healthy controls, the final PCR fragments of p53 exon 7 with 236 bp in size were digested by HaeIII restriction enzyme. The recognition site GCCC for HaeIII restriction enzyme in wild-type sequences of exon 7 represents the arginine-encoding allele in codon 249 and generates a separated 91 bp fragment beside the fragment of 66 bp and also fragments of 37 bp, 30 bp, and 12 bp. However in individuals with the asp-arginine (each amino acid except for arginine) a distinct fragment of 157 bp plus the fragments of 37 bp, 30 bp, and 12 bp in size has been recognized. Two patients showed the homozygous pattern for the mutation (both alleles were mutated) while the other 14 patients were heterozygous for the same mutation. All the three mutations found in the control populations had heterozygous pattern.

3.2. Mutation Distribution among Patients and Control. The characteristics of all the subjects in two groups of case and control were presented in Table 1. There were a total of 133 patients with mean age of 64±9 years old compared to eighty-five noncancerous individuals with mean age of 64±11 years.
Moreover, earlier study on molecular pathology of are important in G1/S checkpoint machinery and apoptosis genes like TP53 and the mechanisms that are important in G1/S checkpoint machinery and apoptosis [20].

The residue of R249 is one of the most frequent mutation in human cancers which promotes the transition of G0 to G1 and/or M to G1 during the cell cycle and is supported by the mutant form of p53 protein which has a longer half-life [23]. Nonetheless the mutation in this residue is not in direct contact with DNA; however it has an important role in stabilizing the DNA-binding structure of p53 and therefore can be attributed as structural mutant [16, 24]. These types of mutants change the conformation of the protein that affects the overall architecture of the DNA-binding surface and its function [24].

The results obtained by Rui et al. revealed that the carboxyl half of the DNA-binding domain (DBD) of p53, including the residue of R249, is responsible for direct interaction with Axin [25]. This protein and p53 are tumor suppressors that control cell growth, apoptosis, and development [25, 26]. Beside the interaction with p53 via HIPK2 protein, Axin can also directly interact with p53 protein through a separate domain to stimulate p53-dependent reporter transcription [25]. It is possible that the mutation in R249 residue disturb the function of Axin tumor suppressor for facilitating the p53 function. Another possible explanation for the R249 mutation is the dietary exposure to the fungal toxin, the aflatoxin B1 (AFB1). The R249 mutation is more frequent in patients with hepatocellular carcinoma in a region with high levels of AFB exposure in their diet. From general aspect, mutation at codon 249 could increase TP53 mRNA expression and result in overexpression of mutant p53 proteins [27].

Of the sixteen R249 mutations that were found in our patients group, 14 showed positivity in the heterozygous form that exhibit deficiency of one allele which is sufficient for a lethal outcome. It has been proposed that the development of cancers in the exposure to carcinogens is even more accurate and faster when combined with heterozygosity for p53 (deficiency of one allele) [28]. However, because of the source of plasma DNA from different cells, the heterozygous cases could have the homozygous/heterozygous mixed pattern.

| Table 1: Characteristics of the study population. |
|-----------------------------------------------|
| Case (n = 133) | Control (n = 85) | P value |
| Age (mean ± SD) | 64.2 ± 9.6 | 64.7 ± 11.6 | 0.739 |
| Gender (female/male) | 53/80 | 48/37 | 0.016 |
| Smoking (yes/no) | 25/108 | 23/62 | 0.151 |
| Familial history of cancer (yes/no) | 28/105 | 26/59 | 0.112 |
| TP53 mutation at Codon 249 (R249) (n (%)) | 16 (11%) | 3 (3.5%) | 0.030 |

old (P = 0.7) that did not differ between the patients and the controls. However there was significant difference in gender between the patients and the controls (P = 0.016) which was because of lower control sample size. When we matched eighty-five individuals of both cases and control, there was no significant difference in sex between groups. Proportion of smokers also did not show significant difference between two groups. The relative frequency of TP53 mutation at codon 249 was 11.1% (16 of 133 samples) in patients with pancreatic cancer compared to 3.5% (3 of 85 samples) in the group of control which was significant (P = 0.03). There was also no difference in familial history of cancer between two groups (Table 1).

The odds ratios for the mutation of TP53 at codon 249 are shown in Table 2. This mutation demonstrated statistically significant association with pancreatic cancer risk (OR 3.74: 95% CI 1.1–13.2; P = 0.041). Remarkably despite the significant difference in the distribution of gender between the patients and the controls, when R249 mutant was adjusted for other characteristics, there was no significant association with pancreatic cancer risk (adjusted OR: 3.12, 95% CI: 0.84–11.6; P = 0.089). The wide variation of confidence interval and P value could be refined when the sample size of cases and controls was revised.

4. Discussion

In pancreatic cancer, the progression from minimally dysplastic epithelium to invasive carcinoma accompanied with the consecutive accumulation of mutations that include activation of oncogenes and inactivation of the tumor-suppressor genes like TP53 [10, 11, 19]. The results of this study identified the higher frequency of TP53 mutation at codon 249 in pancreatic cancer patients. We found that the R249 mutation increased the risk of cancer with no significant difference in the age and gender at cancer diagnosis.

The results from recent study by Biankin et al., which performed exome sequencing and copy number analysis, defined the significant mutations in 16 genes. Their results not only are reaffirming known mutations in individual pancreatic cancers but also by using GeneGO15 showed the importance of mutations in TP53 and the mechanisms that are important in G1/S checkpoint machinery and apoptosis [20]. Moreover, earlier study on molecular pathology of human pancreatic cancer by Ruggeri et al. with regard to the role of known tumor-suppressor genes revealed missense mutations in codons 181, 220, 248, 249, 265, 272, and 273 and suggested the important features of these mutations in the development of human pancreatic cancer [21]. Mutant TP53 has extra functions above loss of normal activity of p53; this stems from the study by Morton et al. that evidenced the function of TP53 mutations in pancreatic cancer to escape from growth arrest/senescence and a promotion of metastasis [22].

The results obtained by Rui et al. showed that the R249 mutation in the plasma of patients with pancreatic cancer is more frequent in patients with pancreatic cancer than in control subjects (OR 3.74: 95% CI 1.1–13.2; P = 0.041). Remarkably despite the significant difference in the distribution of gender between the patients and the controls, when R249 mutant was adjusted for other characteristics, there was no significant association with pancreatic cancer risk (adjusted OR: 3.12, 95% CI: 0.84–11.6; P = 0.089). The wide variation of confidence interval and P value could be refined when the sample size of cases and controls was revised.

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5. Conclusion

This study showed a higher frequency of R249 mutation in patients with pancreatic cancer. Also the presence of the R249 TP53 mutation in the plasma of patients with pancreatic cancer and also in healthy subjects may reflect chronic exposure to high levels of AFB1. Further studies are needed to better define the role of TP53 alterations in pancreatic cancers and possibly to understand the impact of mutations on cancer prognosis and outcomes.
Table 2: Unadjusted and adjusted odds ratios (OR) and 95% confidence intervals (95% CI) for the TP53 mutation at codon 249 status in cases and control subjects.

|                 | Univariable model |          |          | Multivariable model |          |          |
|-----------------|-------------------|----------|----------|---------------------|----------|----------|
|                 | Unadjusted OR (95% CI) | P value | Adjusted OR (95% CI)* | P value |
| R249 wild type (%) | 1 |          | 1 |          |
| R249 mutant (%)   | 3.74 (1.1–13.2) | 0.041 | 3.12 (0.84–11.6) | 0.089 |

*Adjusted odds ratio for gender, age, smoking, and familial history of cancer.

Conflict of Interests
The authors declare no conflict of interests.

Authors’ Contribution
All authors contributed both to the research and the discussion, and they have read and approved the final paper.

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References
[1] G. G. Schwartz and I. M. Reis, "Is cadmium a cause of human pancreatic cancer?" Cancer Epidemiology Biomarkers and Prevention, vol. 9, no. 2, pp. 139–145, 2000.
[2] Iran MoHaEo, "Iranian annual of national cancer registration report," Tech. Rep., 2008.
[3] A. Taghavi, Z. Fazeli, M. Vaheedi, A. R. Baghestani, M. R. Zali, and M. A. Pourhoseingholi, "Pancreatic cancer mortality and misclassification—bayesian analysis," Asian Pacific Journal of Cancer Prevention, vol. 12, pp. 2271–2274, 2011.
[4] Y. Lin, R. Fu, E. Grant et al., "Association of body mass index and risk of death from pancreas cancer in Asians: findings from the Asia Cohort Consortium," European Journal of Cancer Prevention, vol. 22, no. 3, pp. 244–250, 2013.
[5] H. Manuel, "Pancreatic cancer," New England Journal of Medicine, vol. 362, no. 17, pp. 1562–1617, 2010.
[6] A. L. Mihaljevic, C. W. Michalski, H. Friess, and J. Kleeff, "Molecular mechanism of pancreatic cancer—understanding proliferation, invasion, and metastasis," Langenbeck's Archives of Surgery, vol. 395, no. 4, pp. 295–308, 2010.
[7] S. P. Grekova, A. Angelova, L. Daefler, and Z. Raykov, "Pancreatic cancer cell lines can induce prostaglandin E2 production from human blood mononuclear cells," Journal of Oncology, vol. 2011, Article ID 741868, 5 pages, 2011.
[8] A. Petitjean, M. J. W. Achatz, A. L. Borresen-Dale, P. Hainaut, and M. Olivier, "mutations in human cancers: functional selection and impact on cancer prognosis and outcomes," Oncogene, vol. 26, no. 15, pp. 2157–2165, 2007.
[9] G. C. Kabat, R. A. Kandel, A. G. Glass et al., "A cohort study of p53 mutations and protein accumulation in benign breast tissue and subsequent breast cancer risk," Journal of Oncology, vol. 2011, Article ID 970804, 9 pages, 2011.
[10] T. Hamzehloie, M. Mojarrad, M. Hasanzadeh-Nazarabadi, and S. Shekouhi, "The role of tumor protein 53 mutations in common human cancers and targeting the murine double minute 2-P53 interaction for cancer therapy," Iranian Journal of Medical Sciences, vol. 37, no. 1, pp. 3–8, 2012.
[11] G. H. Sakorafas and V. Smyrniotis, "Molecular biology of pancreatic cancer: how useful is it in clinical practice?" Journal of the Pancreas, vol. 13, pp. 332–337, 2012.
[12] K. O. Wong, B. S. DeDecker, S. M. V. Freund, M. Proctor, M. Bycroft, and A. R. Fersht, "Hot-spot mutants of p53 core domain evince characteristic local structural changes," Proceedings of the National Academy of Sciences of the United States of America, vol. 96, no. 15, pp. 8438–8442, 1999.
[13] G. D’Orazi and D. Givol, "p53 reactivation: the link to zinc," Cell Cycle, vol. 11, pp. 2581–2582, 2012.
[14] K. Szymánska, O. A. Lesi, G. D. Kirk et al., "Ser-249 TP53 mutation in tumour and plasma DNA of hepatocellular carcinoma patients from a high incidence area in the Gambia, west Africa," International Journal of Cancer, vol. 110, no. 3, pp. 374–379, 2004.
[15] O. Suad, H. Rozenberg, R. Brosh et al., "Structural basis of restoring sequence-specific DNA binding and transactivation to mutant p53 by suppressors," Journal of Molecular Biology, vol. 385, no. 1, pp. 249–265, 2009.
[16] G. D. Kirk, O. A. Lesi, M. Mendy et al., "249ser TP53 mutation in plasma DNA, hepatitis B viral infection, and risk of hepatocellular carcinoma," Oncogene, vol. 24, no. 38, pp. 5858–5867, 2005.
[17] G. C. Kimbi, M. C. Kew, M. C. Yu, K. Arakawa, and J. Hodkinson, "249ser p53 mutation in the serum of black southern African patients with hepatocellular carcinoma," Journal of Gastroenterology and Hepatology, vol. 20, no. 8, pp. 1185–1190, 2005.
[18] T. Lecomte, A. Berger, F. Zinzindohoué et al., "Detection of free-circulating tumor-associated DNA in plasma of colorectal cancer patients and its association with prognosis," International Journal of Cancer, vol. 100, no. 5, pp. 542–548, 2002.
[19] J. Sullivan, Q. Gong, T. Hyslop et al., "Serum monocyte chemoattractant protein-1 in pancreatic cancer," Journal of Oncology, vol. 2011, Article ID 518394, 6 pages, 2011.
[20] A. V. Bienkin, N. Waddell, K. S. Kassahn et al., "Pancreatic cancer genomes reveal aberrations in axon guidance pathway genes," Nature, vol. 491, pp. 399–405, 2012.
[21] B. Ruggeri, S.-Y. Zhang, J. Caamano, M. DiRado, S. D. Flynn, and A. J. P. Klein-Szanto, "Human pancreatic carcinomas and cell lines reveal frequent and multiple alterations in the p53 and Rb-1 tumor-suppressor genes," Oncogene, vol. 7, no. 8, pp. 1503–1511, 1992.
[22] J. P. Morton, P. Timpson, S. A. Karim et al., "Mutant p53 drives metastasis and overcomes growth arrest/senescence in pancreatic cancer," Proceedings of the National Academy of Sciences of the United States of America, vol. 107, no. 1, pp. 246–251, 2010.
[23] K. P. Vijayaraman, M. Veluchamy, P. Murugesan, K. P. Shanmugiah, and P. D. Kasi, "p53 exon 4 (codon 72) polymorphism and exon 7 (codon 249) mutation in breast cancer patients in southern region (Madurai) of Tamil Nadu," *Asian Pacific Journal of Cancer Prevention*, vol. 13, no. 2, pp. 511–516, 2012.

[24] S. N. Rodin and A. S. Rodin, "Strand asymmetry of CpG transitions as indicator of G1 phase- dependent origin of multiple tumorigenic p53 mutations in stem cells," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 95, no. 20, pp. 11927–11932, 1998.

[25] Y. Rui, Z. Xu, S. Lin et al., "Axin stimulates p53 functions by activation of HIPK2 kinase through multimeric complex formation," *EMBO Journal*, vol. 23, no. 23, pp. 4583–4594, 2004.

[26] R. Najjar Sadeghi, B. Damavand, M. Vahedi et al., "Detection of p53 common intron polymorphisms in patients with gastritis lesions from Iran," *Asian Pacific Journal of Cancer Prevention*, vol. 14, no. 1, pp. 91–96, 2013.

[27] X. M. Peng, W. W. Peng, and J. L. Yao, "Codon 249 mutations of p53 gene in development of hepatocellular carcinoma," *World Journal of Gastroenterology*, vol. 4, no. 1–6, pp. 125–127, 1998.

[28] E. M. Hoogervorst, C. T. M. van Oostrom, R. B. Beems et al., "p53 heterozygosity results in an increased 2-acetylamino- fluorene-induced urinary bladder but not liver tumor response in DNA repair-deficient Xpa mice," *Cancer Research*, vol. 64, no. 15, pp. 5118–5126, 2004.