Mutations in the PTEN/MMAC1 Gene Associated with Cowden Disease and Juvenile Polyposis Syndrome

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Abstract

Objective: In this study, we evaluated PTEN mutations in Cowden Disease and Juvenile Polyposis syndrome. PTEN mutations were detected, cancer and other phenotypes associated with each of these mutations were characterized and loss of wild type PTEN allele in the associated tumors was demonstrated.

Methods: Out of ۹ patients included in this study, ۸ had Juvenile polyposis and ۱ had Cowden syndrome. PTEN gene was evaluated by means of polymerase chain reaction, single strand conformation polymorphism (SSCP), Heteroduplex mobility assay (HMA) and direct DNA sequencing.

Results: According to the results of this research, nucleotide substitutions in PTEN gene were found in ۲۲٪ (۲/۹) of patients. The samples were found to be heterozygote for the c.۳٤۱T>G and c.۳۸۹G>A mutations. One novel mutation c.۳٤۱T>G in Iranian patients with Cowden syndrome was found in this study.

Conclusions: The study of these rare patients could provide insight into PTEN driven tumorgenesis.

Keywords: cowden syndrome; juvenile polyposis syndrome, PTEN gene

Introduction

Cowden Disease (CD, MIM ۱۵۸۳۵۰) is an autosomal dominant genetic disease whose incidence is still not precisely defined. Mutations in the PTEN gene on ۱۰q۲۳,۳ are associated with ۸۵ percent of CD, and ٤۰–۵۰ percent of cases are sporadic. Cowden Disease is characterized by the presence of multiple trichilemmomas, mucocutaneous papillomatosis, acral keratosis, as well as the development of hamartomas and benign tumors in a variety of tissues including lesions in the breast (fibrocystic disease, and cancer) and thyroid gland (goiter, adenoma, and carcinoma) [۱،۲]. More than ۹۰ percent of individuals affected with CD are believed to manifest a phenotype by the age of ۲۰ years. By the end of the third decade (i.e. ۲۹ years), ۹۹ percent of the affected individuals are believed to have developed at least the mucocutaneous signs of the syndrome. The most commonly reported manifestation of CD in women is an increased risk of developing breast cancer, and nearly all women with CD develop bilateral fibrocystic disease of the breast [۳].

Juvenile polyposis syndrome (JPS, MIM ۱۷٤۹۰۰) is an autosomal dominant hamartoma polyposis syndrome. The incidence of JPS has been estimated to range between one in ۱٦،۰۰۰ and one in ۱۰۰،۰۰۰ [٤]. Juvenile polyposis syndrome (JPS) is characterized by predisposition to hamartomatous polyps. Most juvenile polyps are benign; however, malignant transformation can occur [۵]. The term "juvenile" refers to the type of polyp rather than to the age of onset of polyps. Most individuals with JPS have some polyps by ۲۰ years of age. Histopathology plays a critical role in the diagnosis of JPS (figure ۱). While there were early reports of PTEN mutations in “JPS”, these were not confirmed. These individuals either had features suggestive of Cowden syndrome or were too young for the manifestations of Cowden syndrome to be apparent. In one study, ۵۵ individuals in whom JPS was diagnosed were examined and one was found to carry a PTEN mutation [٦]. JPS individuals found to carry PTEN mutations should in fact be reclassified as Cowden syndrome or PTEN Hamartoma Tumour syndrome (PHTS).

The protein tyrosine phosphatase and tensin homologue PTEN is a tumor suppressor of glioblastoma, breast cancer and prostatic cancer [۷،۸]. The gene has at least three names: "PTEN" (phosphatase with tensin homology), "MMAC۱" (mutated in multiple advanced cancers), and "TEP۱" (TGFB-regulated and epithelial cell enriched phosphatase). In primary breast carcinomas, both somatic and germ-line PTEN mutations occur [۹،۱۰]. PTEN contains ۹ exons and encodes a ٤۰۳ amino acid lipid phosphatase that dephosphorylates D۳ of phosphatidylinositol (۳،٤،٥) trisphosphate (PtdIns (۳،٤،٥) P۳), producing phosphatidylinositol (٤،٥) bisphosphate (PtdIns (٤،٥) P۲) acting in opposition to phosphatidylinositol ۳ kinase (PI۳K) [۱۱].

In this study, mutation of PTEN was analyzed in ۹ individuals originally from Isfahan suspected to have Cowden syndrome or were diagnosed with Juvenile polyposis syndrome. The diagnosis of Cowden syndrome and Juvenile polyposis syndrome was made on the basis of patient's particular clinical history and pathological evidence.

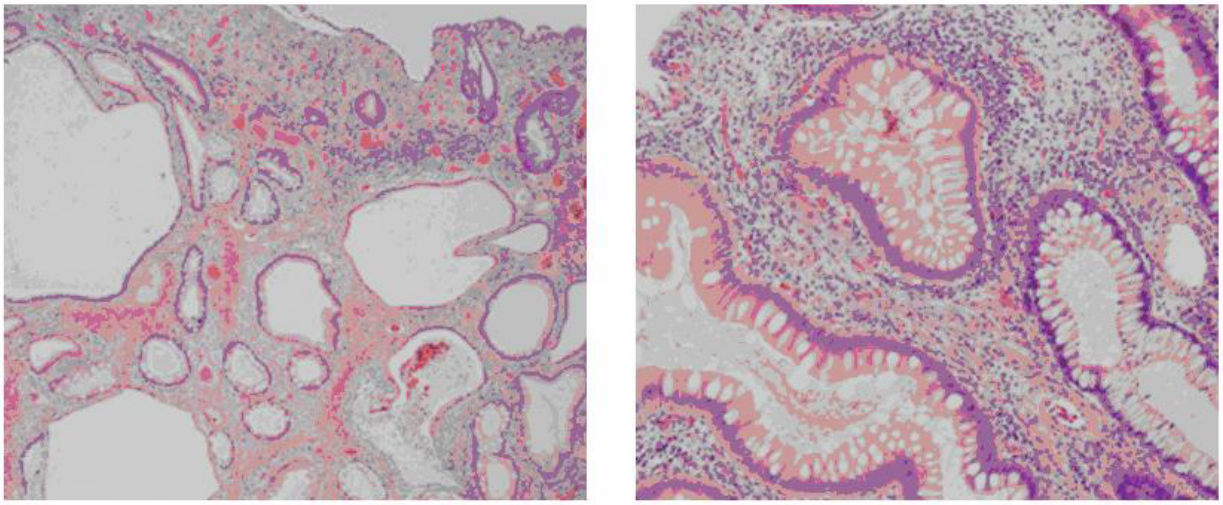
Because these patients are at higher risk of developing malignancies and have no established medical therapies, early screening, surveillance, and preventive care are important issues. Inhibitors of the PI۳K/Akt/mTOR pathway such as rapamycin and its derivatives, which are being developed as cancer therapeutics, could provide new therapeutic options for these rare patients. Rapamycin, also named sirolimus, was isolated from S. hygroscopicus in ۱۹۷۲ [۱۲]. Inhibition of tumour growth with Rapamycin derivatives CCI-۷۷۹ (Wyeth), RAD۰۰۱ (Novartis), AP۲۳٥۷۳ (Ariad) is induced through loss of the PTEN.

Material and Methods

Specimens and isolation of DNA

Nine resected specimens which were primary confirmed by histology were obtained from Seyed Al shohada Hospital in Isfahan, one individual with Fibrocystic Breast Disease suspected to have Cowden syndrome and ۸ individuals diagnosed with Juvenile polyposis syndrome. The diagnosis was made on the basis of patient's particular clinical history and pathological evidence. The tissues were frozen immediately after surgical resection and were stored at -۲۰ ºC until further study. Tumors were dissected to eliminate normal tissue contamination as clean as possible. Genomic DNA in Tissues samples was isolated by standard procedure and was used for the PCR reactions [۱۳].

The following clinical and biological parameters were found in these patients: age ranged from ۱۷ to ٥۳ years (mean ٤۰,۸ years); histological types: ٤ infiltrating ductal carcinomas, ۳ intraductal carcinomas, and ۱ medullary carcinoma; ۲۲٪ had lymph node metastasis. None of the cases had a family history of breast cancer. According to the TNM classification, the clinical stages found in these cases were as follows: stage I: ٤ tumors; stage II: ۳ tumors, and unclassified: one tumor.



A B

Figure ۱. Histology of resected polyps. A: Sessile peduncolated lesion with features of a typical juvenile polyp, characterized by large cysts containing mucus. B: Another polypoid lesion with features of juvenile polyp.

Detection of PTEN gene mutations

Screening for mutations was done in exons ٥, ٨ and ٩ of PTEN gene by PCR-SSCP analysis, Heteroduplex mobility assay (HMA), and DNA sequencing. DNA concentration was measured by OD٢٦٠, and purity was checked by the ratio of OD٢٦٠/OD٢٨٠.

PCR analysis

In order to detect PTEN mutations, exons ٥, ٨ and ٩ PCR amplification was performed in total volume of ٢٥µl with each set of primers for ٣٥ cycles in a DNA thermal cycler. Each cycle included denaturation at ٩٤ ºC for ٣٠ seconds, annealing for ١ minute at the temperature shown in Table ١ and extension at ٧٢ ºC for ١ minute. The five microliters amplification products were fractionated on a ٢٪ agarose gel and visualized by ethidium bromide staining and UV transillumination (fig ٢).

Single strand conformation polymorphism (SSCP) analysis

After PCR amplification of DNA samples from affected and unaffected individuals, the products of each reaction were diluted ١:١ with Gel loading buffer containing ٢٠ mM EDTA, ٠,٢٥٪ Bromophenol blue, ٠,٢٥٪ xylene cyanol FF and ٣٠٪ glycerol in distilled water, denatured for ٥ minutes at ٩٥ºC, and then immediately transferred to ice for ٢ minutes until gel application. Each denatured single strand DNA molecule is assumed to have a three dimensional conformation that is dependent on its primary nucleotide sequence. Ten µl of the mixture was then separated in a vertical ١٠٪ non-denaturing polyacrylamide gel. The conditions of electrophoresis were as follows: separation distance ١٥ cm, voltage ١٧٥ V, separation time ٢٠ hours, temperature ٤ºC in ١ ×TBE buffer. After electrophoresis, gel was stained by a silver staining method and viewed on a light box [١٤]. The migration patterns of different single strand DNAs in nondenaturating polyacrylamide gel were compared with the normal sample. SSCP can detect about ٩٠٪ of single base pair mutations in PCR products that are about ٢٠٠bp or less (fig ٣) [١٥].

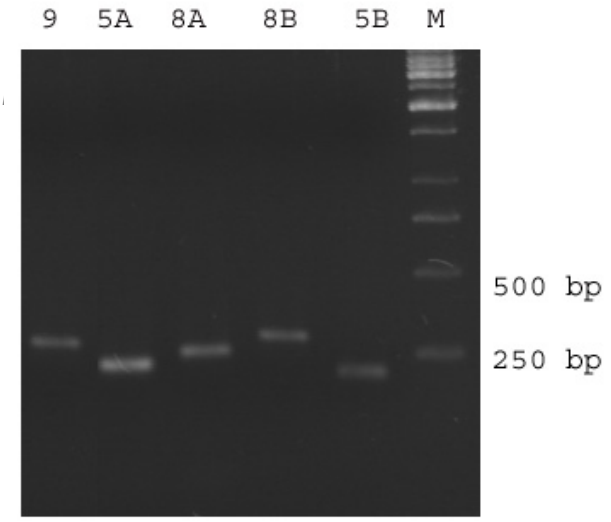


Figure ۲. PCR products on agarose gel. ۵ µl of each PCRs reaction was loaded on a ۲٪ agarose gel and stained with ethidium bromide. Each lane is depicted with the corresponding PCR product name (Lane M: ۱kb Ladder DNA Marker).

Heteroduplex mobility assay (HMA) analysis. In order to confirm the results of Mutations screening by SSCP, we took advantage of HMA analysis. After PCR, ۱۰µl of amplified suspected samples were mixed with ۱۰µl of the normal sample. The mixtures were heated at ۹۸ºC for ۵ minutes and at ۹۰ºC for ۵ minutes, and were then slowly cooled. This method maximizes heteroduplex formation and prepared mixture was maintained for ۲۵ minutes until gel application. The resulting heteroduplexes and homoduplexes in ۱۰µl of sucrose solution (40% sucrose, 0.25% bromophenol blue dye) were separated in a vertical 10% polyacrylamide gel at 215V for 18 hours at room temperature that enhances the difference in mobility between homoduplex and heteroduplex DNA molecules. The gel contained bands with heteroduplex and homoduplex DNA molecules was stained by a silver staining method, and viewed on a light box. This method allows for the observation of imperfectly hybridized heteroduplexes, and can detect more than ۹۵٪ of the single nucleotide mismatches in DNA fragment of ۳۰۰bp or less in length (fig ٤) [۱٦].

Sequencing: For exon ۵ with abnormal SSCP and HMD patterns, the corresponding PCR products were sequenced by the cycling sequencing method in an automatic sequencer. Each sample was sequenced in forward directions. The DNA was extracted from agarose gel by DNA Extraction Kit (K۰۵۱۳).

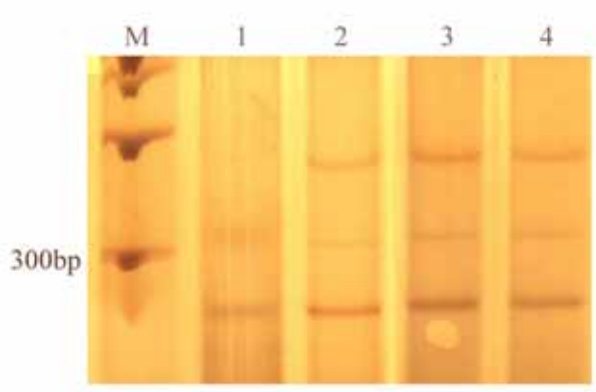


Figure ۳. Results of SSCP analysis for exon ۵A. Lanes ۱-۳: Patients samples, Lane ۱: codon ۱۱۱ mutation showed the shifted bands, lanes ٤: normal sample, Lane M: ۱۰۰bp Ladder DNA Marker.

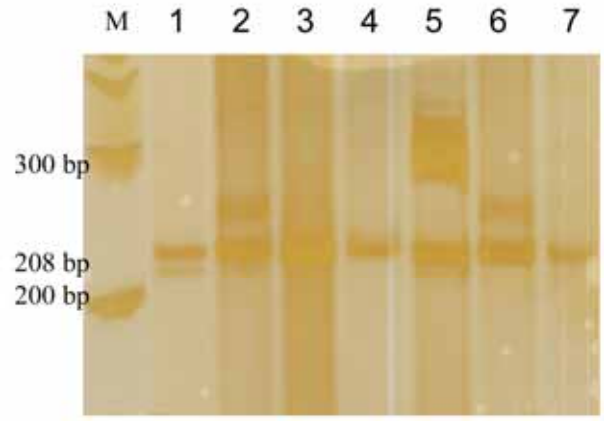


Figure ٤. Heteroduplex mobility analysis (HMA) used to confirm changes in samples showed shifted bands from exon ۵ in SSCP. Lanes ۱ contains the normal sample. Lanes ۲-۷ contain the suspected samples. Heteroduplex bands with mobility shifts in lanes ۲, ۵ and ٦ are mutant samples (M: ۱۰۰bp Ladder DNA Marker).

Results

In this study, after screening for mutations, two patients showed mutations in PTEN gene. We report one novel PTEN mutation in CD, and demonstrate that PTEN mutations are associated with CD and JPS. All identified mutations were present in exon ٥. The samples seem to be heterozygote for the p.R١٣٠Q and p.W١١١G mutations (fig ٥). The novel findings included missense mutations (T→G) at nucleotide 1135 resulting in a tryptophan to glycine change at codon ١١١ (W١١١G). The alignment of the human PTEN gene with homologous genes from xenopus and drosophila indicate that two residues are highly evolutionarily conserved, implying an important functional role for these residues in the PTEN protein. The ideal method to assess the significance of missense mutations would include functional analysis of the translated protein. Examination of missense mutations in codons ١٣٠ revealed that these PTEN missense mutations cause total loss of PTEN function [١٧]. We also evaluated the impact of the observed new missense mutation on the secondary structure of the PTEN protein using MacVector software version ٧,٠. The protein analysis suggested that this mutation result in a detectable change of the surface accessibility of the protein in a region surrounding the altered residues. Taking together the bioinformatics profiling of new mutation and the functionality of the known missense mutation, these missense mutations would be pathogenic.

Case ١. Fibrocystic Breast Disease in a ١٦-Year-Old Female Suspected to Have Cowden Syndrome:

A sample of breast tissues from a ١٦-year-old Iranian female was forwarded for pathological evaluation. She had undergone surgery (mastectomy) for fibrocystic disease of the breast. This patient was asked to return for a meticulous physical examination and pathognomonic cutaneous features of CD were noted. In retrospective review of medical records, it became obvious that this person probably had

Table ١. Primers sequence, length and melting temperature used for the SSCP, HMD and sequencing of the PTEN/MMAC ١ gene.

Primer		sequence	Length of product	Tm
Exon ٥A	F	٥´ACCTGTTAAGTTTGTATGCAAC ٣´	٢٢٩bp	٥٨
	R	٥´TTCCAGCTTTACAGTGAATTG ٣´		
Exon ٥B	F	٥´GACCAATGGCTAAGTGAAGAT ٣´	٢٠٨bp	٥٢
	R	٥´TCCAGGAAGAGGAAAGGAAA ٣´		
Exon ٨A	F	٥´TGCAAATGTTTAACATAGGTGA ٣´	٢٦٢bp	٥٤
	R	٥´GTAAGTACTAGATATTCCTTGTC ٣´		
Exon ٨B	F	٥´AGTCTATGTGATCAAGAAATCGA ٣´	٣٠٠bp	٥٤
	R	٥´TCATCATGTTACTGCTACGTAAAC٣´		
Exon ٥	F	٥´ACCTGTTAAGTTTGTATGCAAC٣´	٣٧٩bp	٥٥
	R	٥´TTTCCTTTCCTCTTCCTGGA٣´		
Exon ٨	F	٥´TGCAAATGTTTAACATAGGTGA٣´	٤٨٥bp	٥٣/٥
	R	٥´TCATCATGTTACTGCTACGTAAAC٣´		
Exon ٩	F	٥´ AAGATGAGTCATATTTGTGGGT ٣´	٢٧١bp	٥٣
	R	٥´ TTTTCATGGTGTTTTATCCCTC ٣´		

Cowden syndrome. Mutation screening revealed p.R۱۳۰Q mutation in exon ۵ of PTEN gene. The detection of the PTEN mutation confirmed her condition, even in the absence of sufficient criteria to make the clinical diagnosis of Cowden syndrome. In this case, signs of CD were subtle and were diagnosed in the context of mutation analysis.

Case ۲. Juvenile Polyposis Syndrome in a ۲۷-Year-Old Female:

The diagnosis was made on the basis of patient's clinical history and pathological evidence which together strongly suggested the clinical diagnosis of Juvenile polyposis syndrome. Mutation screening revealed p.W۱۱۱G mutation in exon ۵ of PTEN gene. According to the rule, JPS individuals with PTEN mutation are reclassified as Cowden syndrome.

Discussion

Cowden syndrome is a rare cancer syndrome with an autosomal dominant pattern of inheritance. CD has typical features such as multiple hamartomas, mucocutaneous lesions (trichilemmomas and papillomatous papules), and involves a high risk of thyroid, endometrium, and breast cancers. Breast cancer is the most frequent malignancy in CD patients, and they have a ۳۰–۵۰٪ lifetime risk of developing breast cancer [۱۸]. Fibrocystic breast disease is frequently found in CD, as diagnosed in our patient. CD is heterogeneous, so the clinical diagnosis is extremely difficult. Clinicians may be able to detect CD patients by evaluating the mutational status of PTEN gene in these individuals.

PTEN has a central role in the regulation of the PI۳K/Akt signaling pathway and early development. Inactivating mutations in PTEN stimulate the growth of many tumors [۱۹]. Loss of PTEN removes the negative regulation on the PI۳K/Akt/mTOR pathway and confers a survival advantage to affected cells [۲۰].

In this research, after structural and functional analysis of PTEN exons and protein, we selected exons ۵, ۸, and ۹ for mutations screening. Although mutations have been reported to be scattered along the entire gene, Nearly half of the PTEN mutations found in CD patients were present on exon ۵, particularly in codon ۱۲۹ and ۱۳۰ which have been considered as the mutation hotspot sites. Exon ۵ (codon ۹۰ to ۱۴۲) is located in the protein tyrosine phosphatase core motif domain [۲۱]. Many mutations and deletions occur in the C-terminal region of PTEN/MMAC۱ cluster in exon ۸ around the poly (A) ۶ stretches and phosphorylation sites [۲۲،۲۳]. Exon ۹ encodes PDZ and PEST motifs. PDZ binding motif interacts strongly with the phosphatase domain [۲۹].

The three exons and splice junctions of the PTEN/MMAC۱ gene were screened for mutations by SSCP and HMA. The primers were chosen in the intron sequences to avoid co-amplification of the Proceed PTEN pseudogene (psiPTEN) on ۹q۲۱ chromosomal region.

Since first CD description, less than ۳۰۰ sporadic cases have been published in the literature [۲۴-۲۷]. There are only few recent additional reports since ۲۰۰۰. Bonneau and Longy reported ۱۱۰ germline PTEN mutations in individuals with CD, Cowden-like disease, juvenile syndrome or Bannayan-Riley-Ruvalcaba [۲۸]. In ۲۰۰۲, Kanaseki et al noted evidence of a ۴ bp deletion in exon ۸ and Staal et al found a missense germ line mutation at codon ۲۳۴ (exon ۷) [۲۹،۳۰]. In ۲۰۰۳, a novel insertion was identified in exon ۷ (insertion of a TAAA in codon ۲۲۱, producing a stop codon) [۳۱]. We identified two mutations of the PTEN gene (p.W۱۱۱G and p.R۱۳۰Q) in Iranian patients with suspected CD. These two mutations are missense mutation in exon ۵, One at codon ۱۱۱ (TGG to GGG, Trp to Gly), and the second at codon ۱۳۰ (CGA to CAA, Arg to Gln). The new PTEN mutation in the Iranian CD patient was present in codon ۱۱۱ of exon ۵.

The diagnosis of CD is confirmed when a PTEN mutation is identified. CD is inherited in an autosomal dominant manner. Because CD is likely to be underdiagnosed, the actual proportion of simplex cases (defined as no obvious family history) and familial cases (defined as two or more related affected individuals) cannot be defined. The majority of CD cases are simplex. Perhaps ۱۰-۵۰٪ of individuals with CD have an affected parent. If a parent of the proband has CD, the risks to siblings are ۵۰٪. Each child of an affected individual has a ۵۰٪ chance of inheriting the mutation and developing CD. Genetic counselling is an important step in helping patients and their relatives to decide on the possible options available. In subject of CD with breast carcinoma more aggressive treatment (bilateral mastectomy) should be offered.

Conclusions

We identified mutations of the PTEN gene in two of the nine Iranian patients with CD and JPS. PTEN molecular genetics testing may be help to precise diagnosis of such cases. Further studies, such as identification of protein expression and function of genes, are needed to prove the precise role of PTEN gene in CD.

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