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Pharmacogenomic Profiling of the PI3K/PTEN Pathway in Sporadic Breast Cancer

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ABSTRACT

Background: Pharmacogenomics is the study of genetic variations among individuals to predict the probability that a patient will respond to single or multidrug chemotherapy. Breast cancer is one of the most common cancers among women worldwide. Treatment of breast cancer by application of biological rationales gives us the ability to match the correct pharmacology to individual tumour genetic profiles. The breast cancers exhibit multiple anomalies in phosphatidylinositol 3 kinase pathways, such as phosphatase and tensin homolog deleted on chromosome TEN loss that can be put in context of therapy with rapamycin analogues. Considering the high incidence of breast cancer in Iran, the potential role of tumor suppressor *PTEN/MMAC1*gene was investigated in Isfahanian breast cancer patients. **Methods:** In this study, *PTEN* was evaluated by means of polymerase chain reaction, single strand conformation polymorphism, Heteroduplex mobility assay and direct DNA sequencing in 72 breast cancer tumors for detection and characterization of mutations. **Results:** According to the results of this research, nucleotide substitutions were found in 5/72 (7%) of samples. The sporadic breast cancer patient was found to be heterozygote for the p.D92N, p.C105W, p.D107N, p.A121P and p.R130Q mutations. One novel mutation, p.D107N, was found in this study. **Conclusion:** Loss of PTEN function in breast cancer can occur either by mutation or reduction of PTEN expression in almost half of sporadic breast tumors. This rate of mutations is an important consideration for novel therapeutic in which biological efficacy is influenced by the activity of PTEN. *Iran. Biomed. J. 13 (2): 79-86, 2009*

Keywords: Breast cancer, Pharmacogenomic, Rapamycin, Somatic mutation

INTRODUCTION

The gene coding for PTEN (phosphatase and tensin homolog deleted on chromosome TEN), also called *MMAC1* was identified and localized to chromosome region 10q23.3 as the tumour suppressor gene [1, 2] responsible for susceptibility to Cowden [3], Lhemitte-Duclos and to Bannayan-Zonana syndromes [4].

PTEN contains 9 exons and encodes a 403-amino acid lipid phosphatase that dephosphorylates D3 of phosphatidylinositol (3,4,5)-trisphosphate [PtdIns (3,4,5)P3], producing phosphatidylinositol [4, 5] bisphosphate [PtdIns(4,5)P2] acting in opposition to phosphatidylinositol 3 kinase (PI3K). PTEN has also been shown to possess tyrosine phosphatase activity *in vitro* [1]. PTEN is a member of the large protein tyrosine phosphatase (PTP) family.

The structure of PTEN consists of an N terminal phosphatase domain followed by an associated C terminal C2 domain [6]. The phosphatase domain contains the active site which carries out the enzymatic function of the protein. C-terminal tail contains a cluster of serine and threonine residues (PEST [The proline, glutamic acid, serine, and threonine] sequence) that becomes phosphorylated in many cells, and a binding site for a group of PDZ domain containing proteins. The C2 domain allows PTEN binds to the phospholipid membrane and dephosphorylation of PI3P [7].

*Corresponding Author; Tel. (+98-311) 793 2477; Fax: (+98-311) 793 2456; E-mail: manoochehr@biol.ui.ac.ir. PI3K, phosphatidyl-inositol 3 kinase; PTEN, phosphatase and tensin homolog deleted on chromosome TEN; PTP, protein tyrosine phosphatase; PEST, proline, glutamic acid, serine, and threonine; mTOR, mammalian target of rapamycin; HMA, heteroduplex mobility assay; LOH, loss of heterozygoty.

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Inactivation of PTEN results in the accumulation of PI3P, activation of a serine/threonine kinase (Akt/Protein kinase B) and other signaling molecules including the mammalian target of rapamycin (mTOR), phosphatidylinositol-dependent kinases and Rho family small GTPases [8]. Activation of Akt by PI3P modulates the activity of a variety of downstream proteins including BAD, Mdm2, p27, and FOXO transcription factors that are related to cell growth and survival. The loss of inhibition of these pathways by PTEN gene inactivation is common and correlates with tumor progression and lymph node metastasis in breast carcinoma [9]. PTEN is a key regulator of progression through the cell cycle by inducing the expression of the p21WAF1, p27KIP1, and p57KIP2, three inhibitors of the cell cycle. PTEN by regulation of the MAPK pathway and dephospho-rylation of focal adhesion kinase modulates cell motility and migration [10]. The tumour suppressor p53 activates the transcription of PTEN and mediates transcriptional repression of the gene encoding the p110 subunit of PI3K, and therefore, functions as a negative regulator of the PI3K signaling pathway [11]. Crosslink between the PI3K/PTEN, p53 and multiple others pathways make PI3K/PTEN pathway as a central integrator of cell signaling networks; therefore, potential targets of numerous drugs. The mTOR was identified in 1994 as the kinase targeted by rapamycin linked to the cellular protein FK506 binding protein [12]. The mTOR with regulation of phosphorylation of several translation proteins, mainly 4E-BP1 and eEF2, modulates translation of specific mRNA [1]. Loss of the tumour suppressor gene *PTEN* and activation of *Akt* induce activity of mTOR pathway [1].

Cancer therapies are now ongoing with mTOR inhibitors such as rapamycin and its derivatives. Rapamycin, also named sirolimus, was isolated from *S. hygroscopicus* in 1972 [15]. The efficacy of P13K/Akt/mTOR pathway inhibitors such as rapamycin derivatives CCI-779 (cell cycle inhibitor 779, Wyeth Pharma, Muenster, Germany), RAD001 (Switzerland´s Novartis), and AP23573 (ARIAD Pharmaceuticals, Inc., USA) are induced by loss of the PTEN activity. The screening of tumour biopsy specimens for PTEN, loss of function by immuno-histochemistry, gene sequencing or DNA microarray provide the biological rationales basis for identification of those cancer patients who most likely to benefit from therapy with mTOR inhibitors.

The purpose of this study was the assessment of the quality and frequency of *PTEN* gene mutations in breast carcinoma and considering the possibility of application of novel therapeutic compounds in which biological efficacy is influenced by the activity level of PTEN for breast cancer patients.

MATERIALS AND METHODS

Patients and samples. The studied material comprised of 72 breast carcinomas samples, 30 blood samples and 5 histologically normal breast tissues. Samples were obtained from Seyed Al Shohada Hospital (Tehran, Iran). The stages of tumor sections were determined by the standard TNM system.

DNA isolation. Genomic DNA was extracted from frozen tissue sample using standard phenol-chloroform extraction procedures and was used for the PCR reactions [16]. Isolation of genomic DNA from peripheral blood lymphocytes was carried out by standard techniques [17].

Detection of PTEN gene mutations. The vast majority of PTEN mutations reported in human cancer occurs in exons 5, 8 and 9 [18]. We screened for PTEN mutations in each of these exons using PCR-SSCP analysis, Heteroduplex mobility assay (HMA) and DNA sequencing.

PCR analysis. In order to detect *PTEN* mutations, exons 5, 8 and 9 PCR amplification was performed in a total volume of 25m l with each set of primers for 35 cycles in a DNA thermal cycler, in which each cycle included denaturation at 94°C for 30 min, annealing for 1 min at the temperature shown in Table 1, and extension at 72°C for 1 min. Products of the PCR were examined using 2% agarose gel.

SSCP analysis. After PCR amplification of DNA samples from affected and unaffected individuals, the products of each reaction were diluted 1:1 with gel loading buffer containing 20 mM EDTA, 0.25% bromophenol blue, 0.25% xylene cyanol FF and 30% glycerol in distilled water, denatured at 95° C for 5 min, and then immediately transferred to ice for 2 min until gel application. Each denatured single strand DNA molecule assumes to have a three-dimensional conformation that is dependent on its primary nucleotide sequence. A volume of 10 ml

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Table 1. Primers sequences, length and melting temperatures used for the SSCP, HMD and sequencing of the PTEN/MMAC1 gene.

Tm		Length of product	Sequence (bp)	Primer
Exon 5A	F R	5′ACCTGTTAAGTTTGTATGCAAC 3′ 5′TTCCAGCTTTACAGTGAATTG 3′	229	58
Exon 5B	F R	5′GACCAATGGCTAAGTGAAGAT 3′ 5′TCCAGGAAGAGGAAAGGAAA 3′	208	52
Exon 8A	F R	5′TGCAAATGTTTAACATAGGTGA 3′ 5′GTAAGTACTAGATATTCCTTGTC 3′	262	54
Exon 8B	F R	5′AGTCTATGTGATCAAGAAATCGA 3′ 5′TCATCATGTTACTGCTACGTAAAC3′	300	54
Exon 5	F R	5′ACCTGTTAAGTTTGTATGCAAC3′ 5′TTTCCTTTCCTCTTCCTGGA3′	379	55
Exon 8	F R	5′TGCAAATGTTTAACATAGGTGA3′ 5′TCATCATGTTACTGCTACGTAAAC3′	485	53/5
Exon 9	F R	5′ AAGATGAGTCATATTTGTGGGT 3′ 5′ TTTTCATGGTGTTTTATCCCTC 3′	271	53

of the mixture was then separated in a vertical 10% non-denaturing polyacrylamide gel. The conditions of electrophoresis were as follows: separation distance 15 cm, voltage 175 V, separation time 20 h, temperature 4°C in 1 × TBE buffer. After electrophoresis, gel was revealed by a silver staining method and viewed on a light box [19]. The migration patterns of different single-strand DNA sequences in non-denaturating polyacrylamide gel were compared with normal sample. SSCP can detect about 90% of single base pair mutations in PCR products that are about 200 bp or less (Fig. 1) [1].

HMA analysis. For confirmation results of mutations screening with SSCP, we have taken advantage of HMA analysis. After PCR, 10 µl of amplified suspected sample was mixed with 10 µl of the normal sample. The mixture was heated at 98°C for 5 min and 95°C for 5 min, and slowly cooled. This method maximizes heteroduplex formation and prepared mixture was maintained at 25°C until gel application. The resulting heteroduplexes and homoduplexes in 10 µl of sucrose solution (40% sucrose, 0.25% bromophenol blue dye) were separated in a vertical 10% polyacrylamide gel at 215V for 18 h at room temperature that enhances the difference in mobility between homoduplex and heteroduplex DNA molecules. Under this condition, both of the homoduplex molecules will migrate to the same extent because they have the same conformation and length. The heteroduplex DNA molecules will migrate more slowly through a gel, and, often both migrate at the same rate to form a single band. 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 permits for observation of imperfectly hybridized heteroduplexes and can detect more than 95% of the single nucleotide mismatches in DNA fragment of 300 bp or less in length (Fig. 2) [21].

Sequencing. For exon 5 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 agarose gel band DNA was extracted with DNA extraction Kit (Cinnagen, Iran) according to the manufacturer's protocol.

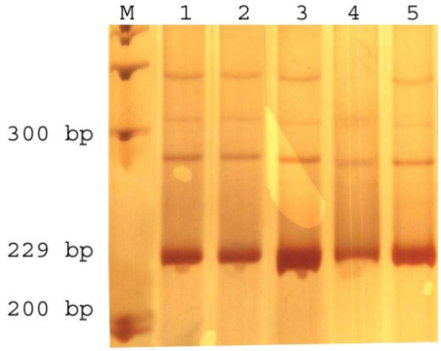

Fig. 1. Representative results of SSCP analysis for exon 5A. lane 1, normal clinical sample; lane 4, codon 92 mutation showed the shifted bands and lanes 2-5, breast cancer clinical samples (M, 100 bp ladder DNA marker)..

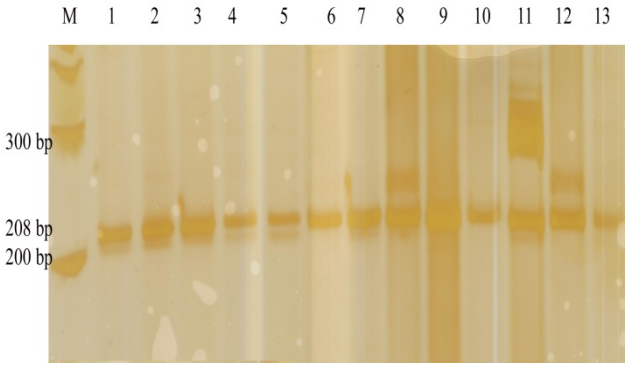

Fig. 2. Heteroduplex mobility analysis was used to confirm change in samples shown the shifted bands from exon 5 in SSCP. Lane 1 contains the normal sample; lanes 2-13 contain the breast cancer and suspected samples. Heteroduplex bands with mobility shifts in lane 8, 11 and 12 are mutant samples (M, 100 bp ladder DNA marker).

RESULTS

Tissue samples from 72 patients with sporadic breast carcinoma who underwent surgical resection were analyzed for *PTEN* gene mutation. Mutations in tumor samples were searched by SSCP analysis. Five of the tissue samples showed aberrant patterns in exon 5. Some suspected samples with or without shift in SSCP bands were also analyzed with HMA. The results of mutations screening by SSCP were confirmed by HMA. Upon sequencing, these shifted bands were found to be due to missense mutations. Normal samples did not show aberrant patterns in SSCP and HMA gel.

Mutations in PTEN gene was observed between codons 92 and 130 in exon 5. The samples seem to be heterozygote for the p.D92N, p.C105W, p.D107N, p.A121P and p.R130Q mutations. The novel findings included missense mutations (G→A) at nucleotide 1123 resulting in an aspartic acid to asparagines change at codon 107 (D107N) (Fig. 3).

The alignment of the human *PTEN* gene with homologous genes from xenopus and drosophila indicated that five residues altered in sporadic breast cancer patients in this study are highly evolutionarily conserved, implying an important functional role for these residues in the PTEN protein (Fig. 4).

The ideal method to assess the significance of missense mutations would include functional analysis of the translated protein. Examine missense mutations in codons 105, 107, 121, and 130 revealed that these *PTEN* missense mutations cause a total loss of PTEN function and that all of them had no effect on the membrane binding activity of protein [22]. Asp-92 of the PTEN WPD loop is in position

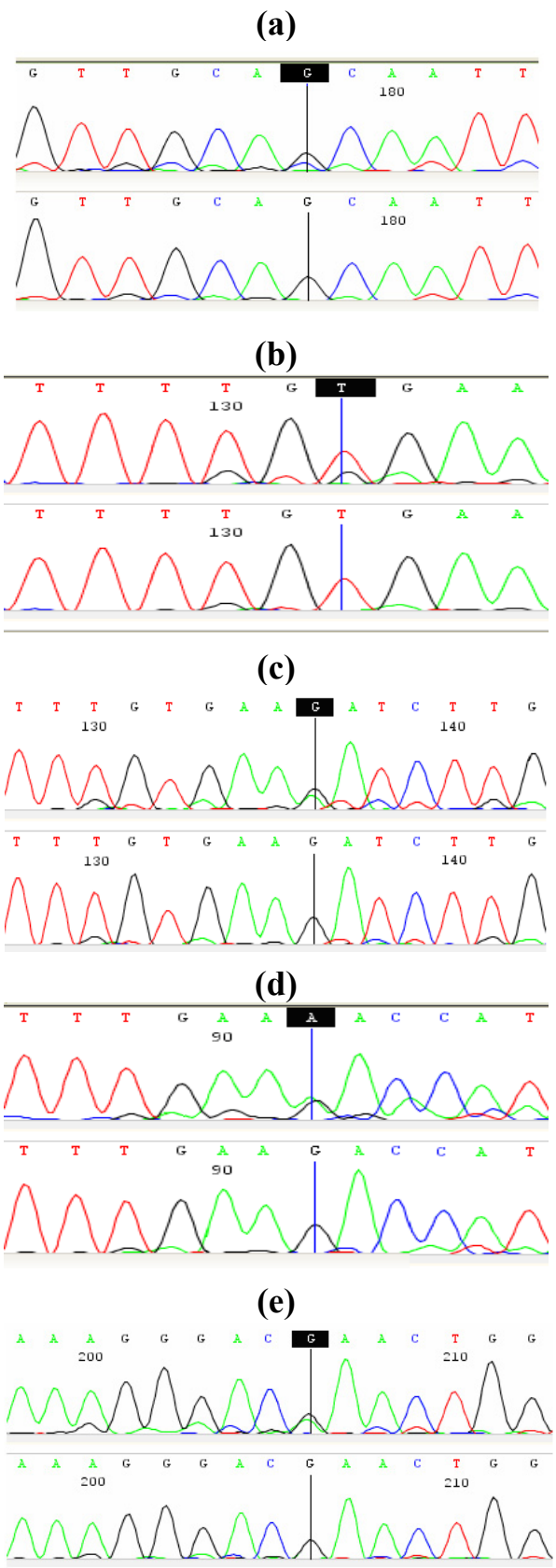

Fig. 3. DNA Sequencing of the exon 5 for Mutation Analysis. **(a)** c.361G>C, **(b)** c.315T>G, **(c)** c.319G>A, **(d)** c.274G>A, **(e)** c.389G>A.

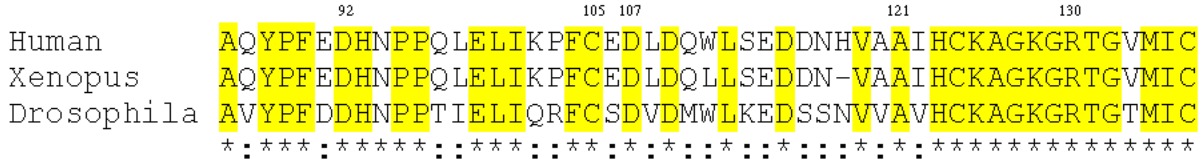

Fig. 4. Multiple alignment of PTEN in human, xenopus and drosophila homologous proteins show the extent of sequences conservation in exons 5 (codon 85-164). Mutations of the six residues (D92, C105, D107, W111, A121 and R130) implicated in sporadic breast cancer patients are conserved among other gene families (shown with codon number).

as the corresponding PTP1B Asp181, which serves as a general acid to facilitate protonation of the phenolic oxygen atom of the tyrosyl leaving group in active site. Similar to the PTP, missense mutation of Asp92 in PTEN results in a 700-fold reduction in catalytic activity suggest that PTEN binds and hydrolyses the phosphate ester with a mechanism similar to that of PTP1B [6].

We also evaluated the impact of the observed new missense mutations on the secondary structure of the PTEN protein using MacVector software version 7.0. The protein analysis suggested that this mutation resulted 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 mutations, these missense mutations would be pathogenic.

In the second part of the study, the histological type and grade of all breast tumor sections were compared with the frequency of *PTEN* gene mutations. Tumors were staged according to the TNM classification and graded according to the World Health Organization recommendations. All Mutations were found in primary breast cancer tumors including ductal *in situ* carcinoma, lobular *in situ* carcinoma, and stage I and II tumor histology (Table 2). Breast cancer patients were all between 22 and 57 years of age. In patients with carcinomas below 45 years, PTEN gene mutations occurred more frequently when compared to carcinomas of the patients over 45 years (59.4 vs. 40.6%).

DISCUSSION

Of 72 primary breast cancer screened 5 (7%) showed mutations in PTEN gene. All identified mutations were present in exon 5. The sporadic breast cancer tumors were found to be heterozygote for the p.D92N, p.C105W, p.D107N, p.A121P and p.R130Q mutations. In this study, one novel somatic mutation within exon 5 of the tumor suppressor gene, *PTEN*, was reported (Fig. 3). The mutations we observed occur in evolutionarily conserved positions (Fig. 4).

PTEN has central role as a negative regulator of the ubiquitous PI3K/Akt signaling pathway. Many tumor growths are stimulated by activating somatic mutations in PIK3CA and inactivating somatic mutations in *PTEN*. The frequency of PTEN inactivation in cancer, include mutation, epigenetic silencing of promoter and post translational modification has continued to rise as new insights into the pathology of its inactivation in cancer [23]. Mutations in the various components of the PI3K/PTEN/Akt pathway have been lead to the acquisition of all common features of the transformed cell [24], so, this pathway is an excellent target for cancer therapy. Breast tumors which exhibit multiple anomalies in PI3K/PTEN/Akt pathway, such as PTEN loss, can now be put in context of therapy with rapamycin analogues, direct inhibitors of the PI3 kinase catalytic subunit and mTOR. Patients with PTEN-deficient breast cancers also had significantly poorer responses to trastuzumab-based therapy than those with normal PTEN [25]. So, PTEN activity, or lack thereof, can serve a powerful predictor for efficacy of rapamycin analogues and trastuzumab resistance in breast cancer.

Table 2. Tumor, nodes, metastasis system (TNM) classification of mutated samples.

Mutation	TNM classification		
	T	N	M
p.D92N	T2	N1	M0
p.C105W	T2	N1	M0
p.D107N	T3	N0	M0
p.A121P	T2	N0	M0
p.R130Q	T2	N0	M0

T, Tumor size; T2, 2-5 cm; T3, >5 cm; N, node status; N0, clear, or negative nodes; N1, cancerous or positive nodes; M, metastasis; M0, no spread of tumor.

In this research, after structural and functional analysis of *PTEN* exons and protein, we selected exons 5, 8, and 9 for mutations screening. A large proportion of mutations in *PTEN* have been mapped to the exon 5, a region encoding the phosphatase domain (codon 90 to 142). Many mutations and deletions occurring in the C-terminal region of *PTEN* that cluster in exon 8 around the poly(A)6 stretches and phosphorylation sites. Ply(A) stretches exist in codons 321-323 in exon 8 [18]. Carboxyl terminal region is essential for regulating the stability and enzymatic activity of PTEN and that mutations in this region are responsible for the loss of the tumor suppressor activity [26]. Exon 9 encodes PDZ and PEST motifs. PDZ-binding motif interacts strongly with the phosphatase domain [29]. PDZ motif plays an important role in ability of PTEN to inhibit Akt and is significant region for interactions between PTEN and others proteins [27]. PEST sequences or phosphorylation sites are essential for PTEN stability [26].

Mutations in tumor suppressor genes are not always completely recessive. In addition, recessive mutations can be haploinsufficient, dominant negative or gain of function. Inactivation of just one allele in group of tumor suppressor genes including *PTEN* is sufficient to permit the formation of tumor [28]. In mice, *PTEN* haploinsufficiency accelerates tumorigenesis in various cancers [29]. Decreased PTEN activity has also been identified in several human cancers [30]. Observations support the hypothesis that the missense mutations can act as dominant negatives and inactivation of a single allele in diploid cells decreases the probability of gene expression from the remaining allele [31]. Supporting this postulate is the observation that missense mutations forms of PTEN that loosed lipid phosphatase activity increase the resistance of the MCF-7 cells to doxorubicin, a chemotherapeutic drug used in breast cancer therapy, suggesting that these *PTEN* mutants acted as dominant negative mutants to suppress wild type PTEN activity [32]. Immuno-histochemistry studies in tumors where loss of heterozygoty (LOH) is common, but second mutations are rare including breast, demonstrated loss of PTEN protein in more than 50% of samples [33]. Where loss of one allele of *PTEN* may have a partial tumor promoting effect, cooperation between loss of one allele of *PTEN* and epigenetic alterations in the other allele leading to a complete loss of function [34]. This observation provides a potential explanation for the discordance in rates of LOH at 10q23 and biallelic *PTEN* inactivation observed in many human malignancies include mutated sporadic breast cancer specimens in this study. *PTEN* haploinsufficiency results in a malfunction of Fas mediated apoptosis [35]. These results support the notion that *PTEN* haploinsufficiency plays a causal role in sporadic breast cancer tumorigenesis.

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