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RESEARCH ARTICLE

Association of a potential functional mir-520f rs75598818 G > A polymorphism with breast cancer

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Abstract. Some of the single-nucleotide polymorphisms in miRNA genes have been studied to date to find their association with the risk of breast cancer (BC). However, no study has been conducted to investigate the association of the *mir-520f* rs75598818 G > A in BC. In the present study, rs75598818 association with BC in an Iranian population has been investigated, and an *in silico* analysis was performed to predict the function of rs75598818 polymorphism in BC. The rs75598818 was genotyped in 129 BC patients and 144 healthy women, using the PCR-RFLP method. The frequency of alleles and genotypes were considered to find the associations between rs75598818 alleles/genotypes, and BC risk and pathological characteristics of the patients. Statistical analysis showed that the rs75598818 GA genotype was significantly associated with BC (GA versus GG, OR = 0.50, 95% CI: 0.25–0.98, $P = 0.041$), high-stage BC (stage III/IV versus I/II, GA versus GG, OR = 0.27, 95% CI: 0.09–0.81, $P = 0.015$), and HER-2 positive status (GA versus GG, OR = 19.00, 95% CI: 4.64–77.82, $P < 0.001$). Notably, the rs75598818 GA genotype has a negative association pattern since it reduces the risk of BC and high stage BC. Conversely, it increases the risk of HER-2 positivity. Computational results suggested that the rs75598818 polymorphism affects the stability of *mir-520f* stem-loop and as a result *miR-520f-3p* production that is a potential tumour suppressor. A contribution of the *mir-520f* rs75598818 polymorphism to BC had been unexplored before. In the present study, we performed an association study and a bioinformatics approach to evaluate this polymorphism in BC. However, further functional experiments and large-scale association studies with various ethnicities are required to elaborate our findings.

Keywords. advanced breast cancer; bioinformatics; functional SNP; HER-2; miRNA.

Introduction

With more than one million new cases of breast cancer (BC) worldwide, it has been recognized as the most common type of cancer among women. It is a major public and world health issue with a global 14–16% annual death rate (Anderson and Jakesz 2008). Likewise, BC is the most frequent type of malignancy among Iranian women (Salek *et al.* 2016). Genetic factors play an important role in BC development (Bagheri *et al.* 2016).

MicroRNAs (miRNAs) are highly conserved short, single-stranded, and noncoding type of RNAs with ~22 bases in length. They posttranscriptionally suppress gene expression by mostly binding to 3′ untranslated region (UTR) of target messenger RNAs (mRNAs) in the cytoplasm (D'Angelo *et al.* 2016). Notably, higher complementarity of the miRNA::mRNA complex leads to greater suppression (D'Angelo *et al.* 2016). Potentially, one miRNA regulates the expression of several genes and pathways at the same time (D'Angelo *et al.* 2016). Therefore, miRNAs have been known to participate in the control of numerous metabolic pathways, such as cellular growth and differentiation. A miRNA can function either as an oncogene or a tumour suppressor, depending on its target genes (Zhang *et al.* 2007; D'Angelo *et al.* 2016; Mesrian Tanha *et al.* 2016).

Single-nucleotide polymorphisms (SNPs) are the most common type of genetic variations. SNPs may affect expression or function of genes by substituting different amino acids (Marjan *et al.* 2014), influencing

splicing process (Mesrian Tanha *et al.* 2017), and so forth (Srinivasan *et al.* 2016). Hence, SNPs might affect disease susceptibility and patient outcomes. Particularly, SNPs existing in miRNA gene regions can disrupt miRNA expression, maturation, as well as target-binding affinity and specificity (Chen *et al.* 2014). To date, several SNPs located on the pre-miRNAs sequences, including *miR-499a* (rs3746444 A > G) (Omrani *et al.* 2014; Kabirizadeh *et al.* 2016), *mir-603* (rs11014002 C > T) (Hashemi *et al.* 2015), *mir-146a* (rs2910164 C > G) (Mehskat *et al.* 2016), *miR-608* (rs4919510 C > G) (Hashemi *et al.* 2016), and *mir-34b/c* (rs4938723 T > C) (Sanaei *et al.* 2016) have been studied among Iranian BC patients.

MIR520F, also known as *mir-520f*, produces two different mature transcripts; namely *miR-520f-3p* and *miR-520f-5p*. However, there are more supporting pieces of evidence for *miR-520f-3p* expression (Griffiths-Jones *et al.* 2007). *MIR520F* is located on chromosome 19 and was initially detected in 2005 (Bentwich *et al.* 2005). Interestingly, *miR-373* and *miR-520c*, with the same seed regions to *miR-520f-3p*, can suppress cell invasion in a BC cell line (Keklikoglou *et al.* 2012). Similarly, *miR-520f-3p*, as a potential tumour suppressor, may interfere with cell invasion in neuroblastoma (Harvey *et al.* 2015). These findings endorse that *miR-520f-3p* may act as a tumour suppressor.

To our knowledge, no study has investigated an association between *mir-520f* rs75598818 G > A polymorphism and BC risk. For the first time, this case–control study was conducted to test a possible association between rs75598818 polymorphism and susceptibility to BC in an Iranian population. In addition, this research aimed to bioinformatically explore whether the rs75598818 SNP affects the risk of BC development.

Materials and methods

Study population

Our study population consisted of 129 Iranian women with pathologically confirmed BC, and 144 cancer-free controls, matched for age, sex and ethnicity recruited from the Sayed-ol-Shohada Hospital, Isfahan, Iran between January 2011 and December 2016. All study subjects were genetically unrelated Iranian individuals living in Isfahan city and its surrounding areas. BC patients with any history of familial cancer were excluded to minimize any effect of familial mutations. BC patients were classified into three clinicopathological subtypes in relation to the immunohistochemistry (IHC) status of oestrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor-2 (HER2). Cancer-free control samples were retrieved from women experiencing regular health checkup at the same hospital. The sampling procedure was performed randomly without any information about clinicopathological characteristics. Peripheral blood samples were obtained from the participants and collected in EDTA-tubes. The current study was approved by the Institutional Review Board of the Nourdanesh University of Meymeh, Meymeh, Isfahan, Iran. Informed consent was obtained from all participants.

DNA extraction and detection of rs75598818 SNP genotypes

Extraction of genomic DNA from peripheral blood samples was performed using the Prime Prep Genomic DNA Isolation kit (GeNetBio, Chungnam, South Korea) following manufacturer's instructions. Concentration and purity of extracted DNA were evaluated by using the Nano Drop 1000 spectrophotometer (Nano-Drop Technologies, Wilmington, USA). The extracted DNA was collected at −20°C. Genotyping of the *mir-520f* rs75598818 G > A SNP was performed using the polymerase chain reaction-restriction fragment-length polymorphism (PCR-RFLP) method. Briefly, the sequences of forward and reverse primers were 5′GTG CTG GAG CAA GAG GAT CTC3′ and 5′CGG AGC CCA AGA AAT GTA GG 3′, respectively. PCR was carried out in a thermo cycler (ASTEC PC-818; ASTEC, Fukuoka, Japan). The PCR procedure was carried out in a 25 μL final volume, comprising 100 ng genomic DNA, 2.5 mL 10× solution buffer, 4.0 mM $MgCl_2$, 0.2 mM dNTPs, 1 pM of each primer and 0.5 unit *Taq* DNA polymerase (Bioron, Germany). The thermal cycles were 5 min at 94°C for initiation, followed by 35 cycles each of 94°C for 30 s, 60°C for 30 s, 72°C for 30 s, and finally 60°C for 5 min. Next, the PCR product with size of 275 bp was digested by the *Taa*I (*Hpy*CH4III) restriction enzyme (ER1361, Thermo Fisher Scientific, Waltham, USA). The fragment carrying G allele was digested and as a result produced two fragments of 178 and 97 bp; on the other hand, the fragment with the A allele remained undigested (275 bp). Electrophoresis of PCR and digested products were loaded on 5% agarose gel electrophoresis in 1× Tris-borate-EDTA buffer at 100 V and finally stained with the RedSafe Nucleic Acid Staining Solution (20,000×) (Boca Scientific, Boca Raton, USA).

Statistical analysis

Statistical tests were performed using statistical package SPSS 19 software (PASW Statistics, SPSS, Chicago). The categorical and continuous demographic variables and risk factors between BC cases and controls were compared using the Pearson's chi-square and Student's *t*-test, respectively. Hardy–Weinberg equilibrium (HWE) consistency and association tests were examined by the Pearson's chi-square test. Logistic regression models were used to account odds ratios (OR) and related 95% confidence intervals (95% CI). In this study, $P < 0.05$ was considered statistically significant.

Table 1. Associations between mir-520f rs75598818 G > A SNP and BC cases.

rs75598818	BC cases (129)	Controls (144)	OR (95% CI)	*P* value[a]
Genotype				
GG	114	114	1.00	0.041*
GA	15	30	0.50 (0.25–0.98)	–
AA	0	0	–	
Allele				
G	243	258	1.00	0.051
A	15	30	0.53 (0.28–1.01)	

[a]Derived from Pearson's chi-square test.
*Statistically significant

Bioinformatics analysis

According to the miRBase database (http://www.mirbase.org/), the *miR-520f-3p* is the predominant product of the *mir-520f* stem-loop; therefore, it was considered for further analyses (Griffiths-Jones *et al.* 2007). The performed *in silico* analysis has been described previously (Hasanzadeh *et al.* 2016; Mehskat *et al.* 2016). The *miR-520f-3p* targetome was predicted by a miRNA target prediction tool of the miRWalk v. 2.0 which uses 12 different algorithms for miRNA::mRNA prediction (http://zmf.umm.uni-heidelberg.de/apps/zmf/mirwalk2/index.html); hence, it provides a prediction score from 0 to 12 (Dweep and Gretz 2015). Validated targets of the studied miRNA were obtained from the miRTarBase v. 6.0 (http://mirtarbase.mbc.nctu.edu.tw) (Chou *et al.* 2015). Noticeably, the average of prediction scores (the score was obtained from the miRWalk) for validated targets (the targets were obtained from the miRTarBase) was used as the cutoff for predicted targets inclusion. Particularly, related-gene sets to BC were extracted from the online Mendelian inheritance in man (OMIM) database (https://www.ncbi.nlm.nih.gov/omim) (Hamosh *et al.* 2005) to eliminate nonspecific tissue genes. Enrichment analysis of *miR-520f-3p* targetome was conducted by DAVID v. 6.8 database using kyoto encyclopedia of genes and genomes (KEGG) information (Huang *et al.* 2009). The Bonferroni correction of $P < 0.05$ was the cutoff for the enrichment analysis.

The possible impact of the SNP located on the *mir-520f* gene region, rs75598818 n.80G > A, on structure of the *mir-520f* stem-loop with the two alleles was studied using the RNA structure web tool (http://rna.urmc.rochester.edu/RNAstructureWeb/Servers/Predict1/Predict1.html).

Results

Average ages of the case and control populations were 52.88 ± 11.85 and 50.31 ± 12.87 years, respectively. No significant dissimilarity was found between the groups regarding age ($P > 0.05$). The genotype and allele distributions of the *mir-520f* rs75598818 G > A polymorphism in 129 BC cancer patients and 144 healthy controls are shown in table 1. In all samples (controls and cases), frequency of the A allele was 8.2% which is almost consistent with minor allele frequency of the rs75598818 reported by the 1000 Genomes Project Phase 3. The observed genotype frequencies for this polymorphism was in agreement with HWE in cases ($P = 0.483$) and controls ($P = 0.163$). These consistencies possibly validate the genotyping results.

The present work found that the BC population showed a decreased incidence of the A allele for the *mir-520f* rs75598818 compared to the control population. Our results also show a shift in genotypic frequencies in the BC population compared with the healthy group, significantly showing a decrease in the GA genotype and an increase in the GG genotype frequencies (GA versus GG, OR = 0.50, 95% CI: 0.25–0.98, $P = 0.041$) (table 1). This calculation determined that BC risk decreased 0.5-fold in the heterozygous group compared with the GG genotype.

Moreover, as shown in table 2, we analysed associations between the *mir-520f* rs75598818 SNP and a couple of clinicopathological features, including lymph node metastasis, stage, histological grade, oestrogen receptor status, progesterone receptor status and HER-2 status. An inverse significant association was observed between the GA rs75598818 genotype and risk of high stage of BC (stage III/IV versus I/II, GA versus GG, OR = 0.27, 95% CI: 0.09–0.81, $P = 0.015$). Conversely, we found an increased risk of HER-2 positivity for patients carrying the GA rs75598818 genotype (GA versus GG, OR = 19.00, 95% CI: 4.64–77.82, $P < 0.001$). No significant relationship was observed between *mir-520f* rs75598818 genotypes and other clinicopathological features of BC patients ($P > 0.05$).

To understand possible relationship between the *miR-520f-3p* and BC development, enrichment analysis of molecular signalling pathway was performed. The OMIM introduced 933 BC-related genes. Among these genes, 10 genes were experimentally validated as *miR-520f-3p* targets (*TPD52*, *TFAP4*, *KMT2A*, *HIP1*, *GREB1*, *GLCE*, *ATAD2*, *RAD51D*, *CDKN1B* and *SOCS3*). In addition, 89 predicted target genes with an acceptable score (a predicted score of the gene ≥ an average of the predicted scores for

Table 2. Association between A allele carriers and BC characteristics.

Characteristics	GG	GA/AA	OR (95% CI)	*P* value[a]
Lymph node metastasis				
Positive	42	3	0.43 (0.11–1.61)	0.198
Negative	72	12		
Stage				
III/IV	75	6	0.27 (0.09–0.81)	0.015*
I/II	30	9		
Unknown[b]	9	0		
Grade				
III	30	6	1.20 (0.39–3.70)	0.751
I/II	54	9		
Unknown[b]	30	0		
ER				
Negative	12	6	3.17 (0.95–10.58)	0.053
Positive	57	9		
Unknown[b]	45	0		
PR				
Negative	18	6	1.89 (0.59–6.05)	0.280
Positive	51	9		
Unknown[b]	45	0		
HER-2				
Positive	12	12	19.00 (4.64–77.82)	< 0.001*
Negative	57	3		
Unknown[b]	45	0		

[a]Derived from Pearson's chi-square test.
[b]Samples, 15 BC cases, with unknown variable were excluded for deriving OR and *P* value.
*Statistically significant.

Table 3. MiR-520f-3p targetome signalling pathways in BC using KEGG resource.

Rank	Term	BC related target genes	Bonferroni *P*
1	Pathways in cancer	*RUNX1, CASP8, STAT3, EGFR, MLH1, FGFR2, PML, GNAS, CDKN1B, AKT1, JUP, TP53, FGFR1, CXCL12, AKT3, PTCH2, DAPK2*	<0.001
2	Proteoglycans in cancer	*TFAP4, IGF2, AKT3, STAT3, EGFR, CAV1, ESR1, CD44, AKT1, TP53, PDCD4, FGFR1*	<0.001
3	Prolactin signalling pathway	*AKT3, STAT3, TNFRSF11A, FOXO3, SOCS3, ESR1, AKT1*	0.004
4	Central carbon metabolism in cancer	*AKT3, EGFR, FGFR2, AKT1, TP53, FGFR1*	0.031

the validated targets) were added to our list. Therefore, 99 genes were highlighted as predicted/validated BC-related targets of *miR-520f-3p*. Using the nominated *miR-520f-3p* gene targets (99 genes) and the DAVID functional annotation tool it was suggested that the *miR-520f-3p* potentially has an impact on four pathway terms, including 'pathways in cancer', 'proteoglycans in cancer', 'prolactin signalling pathway' and 'central carbon metabolism in cancer' (table 3).

Finally, the RNA structure predictor was utilized to separately predict a secondary structure and free energy of the *mir-520f* stem-loop with the alternative alleles (−52.2 kcal/mol for the G allele, −47.8 kcal/mol for the A allele, ΔΔG: 4.4 kcal/mol) (figure 1). The result suggests that the substitution of the G allele by the A allele breaks a naturally available hydrogen bond between G and C; therefore, it may reduce the *mir-520f* stem-loop stability.

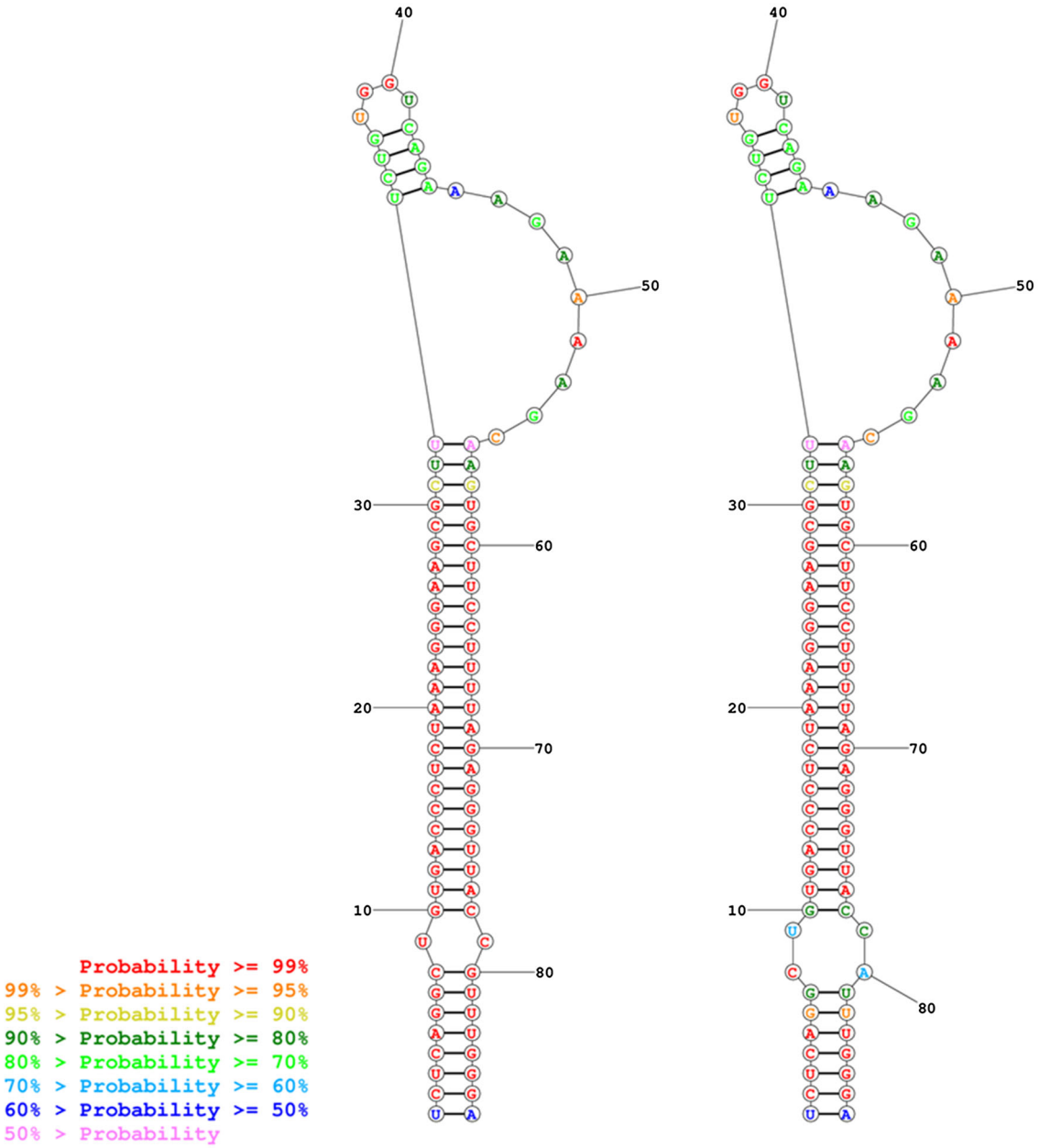

Figure 1. Predicted secondary structure of mir-520f with the G (left) and A (right) allele located in 80th nucleotide. Free energy for the G and A allele was calculated as −5.2 and −47.8 kcal/mol, respectively, by RNA structure web tool.

Discussion

It has been demonstrated that small noncoding miRNAs are crucial components for regulation of gene expression in human cells; hence, abnormal expression of miRNAs is correlated with cancer (Ha and Kim 2014; Mesrian Tanha *et al.* 2016). Inherited single-nucleotide mutations or polymorphisms occurring within miRNA gene regions can alter gene regulation mechanism; thereby, potentially affect the risk of cancer development (Ryan *et al.* 2010). In the present study, we conducted a case–control study to explore the impact of the *mir-520f* rs75598818 n.80G > A variant on the risk of BC in an Iranian population. Moreover, functional bioinformatics assessment was performed to predict a role of this miRNA and rs75598818 SNP in BC development.

Our data indicated that the GA genotype of the rs75598818 SNP, located on the *mir-520f* stem-loop, may offer a reduced risk of BC development (GA versus GG, OR = 0.50, 95% CI: 0.25–0.98, $P = 0.041$). Moreover, the GA genotype was inversely associated with the high stage of BC (stage III/IV versus I/II, GA versus GG,

OR = 0.27, 95% CI: 0.09–0.81, $P = 0.015$). In contrast, the GA rs75598818 genotype was positively associated with HER-2 positivity (GA versus GG, OR = 19.00, 95% CI: 4.64–77.82, $P < 0.001$). Notably, no significant association was found between the rs75598818 G allele and BC risks, and it is possible that a larger population size is needed to detect similar effects for the G allele in general.

To date, a possible relationship between the *mir-520f* and BC susceptibility has been poorly investigated; however, it could be inferred by other miRNAs with a similar seed region. To illustrate, both miR-373 and miR-520c have similar seed regions to the *miR-520f-3p* seed region and suppress cell invasion in a BC cell line. Likewise, Harvey *et al.* (2015) indicated that the *miR-520f-3p* may suppress cell invasion in neuroblastoma. These reports suggest that *miR-520f-3p* may act as a tumour suppressor. To predict a *miR-520f-3p* role in BC we conducted computational investigation for the first time. According to computational results, 'pathways in cancer', 'proteoglycans in cancer', 'prolactin signalling pathway', and 'central carbon metabolism in cancer' are potentially regulated by the *miR-520f-3p* (table 3). Thus, our *in silico* result endorses tumour suppression activity for the *miR-520f-3p* in BC.

The rs75598818 polymorphism (80th nucleotide of the *mir-520f*) is located on neither the *miR-520f-5p* (nucleotides 15–36 of *mir-520f*) nor the *miR-520f-3p* (nucleotides 55–76 of *mir-520f*) mature sequences. This shows that the SNP does not affect miRNA::mRNA binding. On the other hand, bioinformatics result predicted that the rs75598818 SNP alters free energy of the *mir-520f* stem-loop; thus, it likely affects *mir-520f* stability (figure 1). Despite the lack of research into the effects of this SNP in any disease, our result predicts that the A allele reduces the *mir-520f* stem-loop stability and as a result the *miR-520f-3p* production.

In conclusion, this study indicated that the A allele *mir-520f* rs75598818 may reduce production of *miR-520f-3p*, a possible tumour suppressor miRNA in the pathogenesis of BC. In agreement of this hypothesis, the GA genotype is associated with HER-2 positivity. On the other hand, the GA genotype is inversely associated with BC and high-stage BC and this finding can suggest diverse functional aspects for *miR-520f-3p* in normal and cancerous breast cell. Notably, there is no experimental evidence investigating the function of the *miR-520f-3p* and rs75598818 SNP in BC; hence, we cannot have a thorough discussion. Association case–control studies with larger number of samples in various ethnicities are needed to confirm our findings. In addition, further investigations are needed to find an exact role of *miR-520f-3p* in breast normal and cancer cells. Finally, the *miR-520f-5p* was excluded in the present study due to its low expression level in cells based on the miRBase report. However, further studies can be aimed to evaluate the *miR-520f-5p* expression and function in BC.

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