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Distinct prevalence of the CYP19 $\Delta 3(TTTA)_7$ allele in premenopausal versus postmenopausal breast cancer patients, but not in control individuals

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Abstract

The *CYP19* gene encodes the enzyme aromatase, which plays a key role in the conversion of androgens to oestrogens. A polymorphism in *CYP19* in intron 4 (TTTA)n has been reported to be associated with breast cancer (BC) risk, although conflicting evidence has also been published. Here, we employ a non-traditional, highly demonstrative design of a molecular epidemiological study, where the comparison of BC cases and healthy middle-aged female donors was supplemented by an analysis of groups with extreme characteristics of either BC risk (bilateral breast cancer (biBC) patients) or cancer tolerance (tumour-free elderly women aged $\geqslant 75$ years). None of the (TTTA)n polymorphic variants was significantly overrepresented among the affected women compared with any of the control groups. However, a 3-bp deletion/insertion *CYP19* polymorphism, which is located in the same intron approximately 50 bp upstream to the (TTTA)n repeat, was evidently associated with the menopausal status in both the BC and biBC cohorts. In particular, the $\Delta 3(TTTA)_7$ allele occurred significantly more frequently in premenopausal than in postmenopausal BC patients (65/172 (38%) versus 67/310 (22%); $P = 0.0001$; Odds Ratio (OR) = 2.20 (95% Confidence Interval (CI) 1.46–3.32)), while the perimenopausal cases demonstrated an intermediate value (9/34 (26%)). In the biBC cohort, women who developed both tumours during their premenopausal period had a significantly higher prevalence of the $\Delta 3(TTTA)_7$ allele than patients with a postmenopausal onset of bilateral disease (16/46 (35%) versus 8/50 (16%); $P = 0.035$; OR = 2.80 (1.08–7.23)); those biBC patients, whose tumours were diagnosed before and after the cessation of menses, displayed an intermediate occurrence of the $\Delta 3(TTTA)_7$ allele (7/32 (22%)). Similar tendencies in the $\Delta 3(TTTA)_7$ allele distribution in BC and biBC patients suggest that its association with the menopausal status of the patients is truly non-random and thus this observation deserves further detailed investigation.

Keywords: *CYP19*; Polymorphism; Breast cancer; Predisposition; Menopause

1. Introduction

Unfavourable alleles of low-penetrance polymorphic genes appear to play a major role in breast cancer (BC) susceptibility. The list of suspected candidates includes oncogenes (*HRAS-1*), suppressor genes (*TP53*), participants of steroid metabolism and signal transduction (*CYP17, CYP19, COMT, ER, PR*), xenobiotic metabolisers (*GSTM1, GSTT1, GSTP1, CYP1A1, CYP2D6, CYP2E1, NAT2*), and some other variable genetic elements (*HSP70, TNFα*) [1–3]. However, the research of BC polymorphic genes is hampered by a poor reproducibility of the reported effects. The reasons for this are multiple. First, low-penetrance genes are difficult to study by definition, as their effect is weak. Secondly, BC is a very heterogeneous disease, and its pathogenesis

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includes a complex interplay between various hereditary, metabolic, behavioural, environmental and stochastic factors. Therefore, the impact of a given polymorphism may vary between distinct categories of BC patients. Finally, an insufficient study design is frequently blamed for the failure to achieve meaningful conclusions [2,4–6].

Cancer is a very frequent disease that is, in many cases, associated with ageing. Hence, both middle-aged healthy donors and, to a lesser extent, age-adjusted controls, may contain an undefined proportion of potential oncological patients. The share of the latter group could be significant, as the life-time risk of being diagnosed with cancer reaches as high as 48% for men and 38% for women [7]. To overcome this confounding factor, we and others have suggested using additional control cohorts of elderly tumour-free subjects, who have already passed most of their cancer likelihood period and thus represent an example of 'cancer resistance' [8–11]. We also attempted to separate within the groups of sporadic cancer patients those cases which display a particularly high evidence for tumour susceptibility, such as lung cancer (LC) non-smokers for LC [9], or bilateral breast cancer (biBC) patients for BC [10]. This approach, aimed to compare cohorts with extreme characteristics of cancer risk and tolerance, appeared to produce highly demonstrative data [9,10].

A systematic review of BC-predisposing low-penetrance genes emphasised the likely significance of the *CYP19* intron 4 (TTTA)n polymorphism [2]. The *CYP19* gene belongs to the cytochrome family and encodes the enzyme aromatase. Aromatase converts androgens to oestrogens, and definitely plays a role in the development of at least a subset of BC [12,13]. Numerous polymorphisms in the *CYP19* gene have been described. The (TTTA)n repeat polymorphism in intron 4 has attracted the most attention, as Kristensen and colleagues [14] revealed the overrepresentation of the longest $(TTTA)_{12}$ allele in BC patients. This observation have subsequently been confirmed by a study by Haiman and colleagues described in Ref. [15]. In addition, studies by Haiman and colleagues [15], Healey and colleagues [16] and Baxter and colleagues [17] pointed at the possible significance of the $(TTTA)_{10}$ allele. However, conflicting reports have also been published [18,19]. In addition, a 3-bp deletion/insertion polymorphism has been found approximately 50 bp upstream from the (TTTA)n [16,19,20]. Here, we present an analysis of *CYP19* intron 4 alleles in biBC patients, BC patients, healthy middle-aged women and elderly tumour-free females.

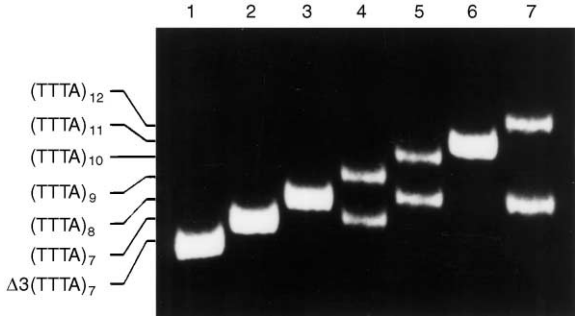

Fig 1. Representative examples of *CYP19* genotypes. Corresponding alleles are marked at the left. The genotypes are $\Delta3(TTTA)_7/\Delta3(TTTA)_7$ (lane 1), $(TTTA)_7/(TTTA)_7$ (lane 2), $(TTTA)_8/(TTTA)_8$ (lane 3), $(TTTA)_7/(TTTA)_9$ (lane 4), $(TTTA)_8/(TTTA)_{10}$ (lane 5), $(TTTA)_{11}/(TTTA)_{11}$ (lane 6), $(TTTA)_8/(TTTA)_{12}$ (lane 7).

2. Patients and methods

2.1. Subjects and DNA isolation

The affected groups included 295 subjects with monolateral BC (BC patients; mean age: 51.4 years; age range: 31–81 years) and 71 cases of biBC (mean age at onset of the first tumour: 50.1 years; age range: 27–72 years; mean age at onset of the second tumour: 54.5 years; age range: 30–80 years). All patients were treated in the N.N. Petrov Institute of Oncology (St. Petersburg, Russia). The tumour-free controls were 117 middle-aged female donors ('healthy women' (HW); mean age: 39.5 years; age range: 18–54 years), who volunteered in the blood transfusion unit of the same Institute, as well as 96 elderly ($\geqslant$75 years old) women ('elderly women' (EW); mean age 78.1 years; age range: 75–90 years) recruited in various hospitals of St. Petersburg. All affected and non-affected subjects were Caucasians of Slavic origin. Peripheral leucocyte DNA was extracted from blood samples from BC patients, HW and EW using the modified salt-chloroform procedure [21]. To accumulate a significant number of biBC cases, we collected paraffin-embedded archival tissues. Specimens from the non-tumour parts of the affected breasts or intact lymph nodes were deparaffinised in xylene and incubated overnight at 60 °C in 100 μl of the lysis buffer (10 mM Tris–HCl, pH 8.3; 1 mM ethylene diamine tetra acetic acid (EDTA); 1% Triton X-100; 500 μg/ml proteinase K). Then the lysates were boiled for 5 min, and diluted 10-fold in water for the subsequent polymerase chain reaction (PCR) analysis.

2.2. CYP19 PCR genotyping

The primers were 5′-GGT ACT TAG TTA GCT ACA ATC-3′ (upstream, nucleotides 610–630) and 5′-GGG TGA TAG AGT CAG AGC CT-3′ (downstream, nucleotides 721–740). The choice of these sequences was predetermined in order to minimise the size of the amplified polymorphic fragment. This design has allowed us to achieve an efficient PCR using partially

degraded archival DNA, as well as an easy separation of alleles of slightly varying length. 10-μl PCR reactions contained 1 μl purified DNA (50–100 ng/μl) or DNA-containing tissue lysate, 0.5 units heat-activated MDP-1 thermostable polymerase (Yours, Moscow), 1× PCR buffer, 1.5 mM $MgCl_2$, 200 μM deoxynucleotide triphosphates (dNTP), 1 μM each primer, and 2% Triton X-100. The addition of the Triton X-100 was found to be absolutely critical for the success of the PCR amplification. The products were separated in the 15% polyacrylamide (30:1) non-denaturing gel by applying the double running distance in the 20 cm vertical gel apparatus (Helicon, Moscow), and visualised by ethidium bromide staining (Fig. 1). The representative allele-specific fragments were eluted from the gel and sequenced by the DNA Sequencing System (Promega) according to recommendations of the manufacturer.

2.3. Statistical analysis

The Chi-square test was used for group comparisons. P values <0.05 were considered statistically significant. Where appropriate, a Mantel–Haenszel analysis of the grouped data and Bonferroni correction for multiple comparisons were performed as well. Odds ratios (OR) were calculated for the standard 95% confidence interval (CI) [22].

3. Results

PCR genotyping allowed us to separate seven different alleles of the *CYP19* gene (Fig. 1). Since the published data concerning the sizes and nucleotide composition of these alleles vary [16,18,20,23], we performed a sequencing analysis of the representative fragments and confirmed that the number of (TTTA)n repeats ranged from 7 to 12. In addition, the fraction of the $(TTTA)_7$ alleles contained a 3 bp deletion in the upstream portion of the fragment. Whereas the sequence of the 127 bp $(TTTA)_7$ variant was 5′-AATCTTCTTTTTTG-3′ (nucleotides 627–640), the 124 bp $(TTTA)_7$ fragment contained 5′-AATCTTTTTTG-3′. It is worth noting that it is impossible to determine precisely which triplet is deleted in the shortest allele, as the loss of either TCT (nucleotides 629–631 or 632–634), or CTT (nucleotides 630–632 or 633–635), or TTC (nucleotides 631–633) would result in the same DNA sequence. The latter observation could explain the apparent controversy between published reports [16,18,20] concerning the nucleotide composition of this 3-bp deletion.

Table 1
CYP19 allele frequencies in bilateral breast cancer patients, monolateral breast cancer patients, healthy middle-aged females, and tumour-free elderly women

CYP19 alleles	biBC ($n=71$)	BC ($n=295$)	HW ($n=117$)	EW ($n=96$)
$\Delta3(TTTA)_7$	36 (25%)	163 (28%)	62 (26%)	64 (33%)
$(TTTA)_7$	28 (20%)	93 (16%)	60 (26%)	23 (12%)
$(TTTA)_8$	26 (18%)	93 (16%)	31 (13%)	31 (16%)
$(TTTA)_9$	0	1 (<0.5%)	0	1 (1%)
$(TTTA)_{10}$	2 (1%)	13 (2%)	2 (1%)	8 (4%)
$(TTTA)_{11}$	47 (33%)	196 (33%)	73 (31%)	58 (30%)
$(TTTA)_{12}$	3 (2%)	31 (5%)	6 (3%)	7 (4%)
Total alleles	142 (100%)	590 (100%)	234 (100%)	192 (100%)

The distribution of *CYP19* alleles and genotypes in biBC patients, BC patients, HW and EW is shown in Tables 1 and 2. Allele frequencies did not differ statistically between any of the groups tested. In addition, none of the genotypes was significantly overrepresented among the affected women (Table 2). Finally, the analysis of the allele carriers (Table 2 and data not shown) also failed to reveal BC-predisposing variants. Comparison of distinct clinical breast cancer variants indicated that *CYP19* polymorphism was not associated with tumour size, nodal involvement or family history (data not shown). However, the $\Delta3(TTTA)_7$ allele was significantly overrepresented in premenopausal BC patients compared with postmenopausal ones (38% versus 22%; $P=0.0001$; OR = 2.20 (95% CI 1.46–3.32)), while the perimenopausal cases demonstrated an intermediate value (26%) (Table 3). Strikingly, a similar tendency was evident in biBC cohort. In particular, women who developed both tumours in the premenopausal period had a higher frequency of the $\Delta3(TTTA)_7$ than patients with postmenopausal onset of the bilateral disease (35% versus 16%; $P=0.035$; OR = 2.80 (1.08–7.23)); those biBC patients, whose tumour diagnoses were separated by the cessation of menses, displayed an intermediate occurrence of the $\Delta3(TTTA)_7$ allele (22%) (Table 3). In agreement with the data on menopausal status, patients aged below 50 years had a higher frequency of the $\Delta3(TTTA)_7$ than older women (BC patients: 38% versus 22%; $P=0.00006$; OR = 2.17 (1.48–3.16); biBC patients: 33% versus 17%; $P=0.059$; OR = 2.39 (0.97–5.86)). Notably, the increased prevalence of the $\Delta3(TTTA)_7$ allele in women of premenopausal age was characteristic only for breast cancer patients, and not for the controls. As seen in Table 1, healthy female donors, who were mainly premenopausal, had a $\Delta3(TTTA)_7$ allele frequency of 26%. In contrast, geriatric controls demonstrated a higher occurrence of the $\Delta3(TTTA)_7$ (33%), although the difference was below the statistical limit ($P=0.124$; OR = 0.72 (0.48–1.09). While the $\Delta3(TTTA)_7$ allele distribution did not possess specific features in randomly selected BC patients (Table 1), comparison of premenopausal cases with HW showed

evidence for an association of this variant with premenopausal BC risk (38% versus 26%; $P = 0.015$; OR = 1.69 (1.11–2.57) (Tables 1 and 3). Conversely, the $\Delta3(TTTA)_7$ allele appeared to have some protective effect in postmenopausal women (postmenopausal BC patients versus EW: 22% versus 33%; $P = 0.004$; OR = 0.55 (0.37–0.82)) (Tables 1 and 3). Taken together, our data provide strong evidence for an association between the CYP19 $\Delta3(TTTA)_7$ allele and the menopausal status of breast cancer patients.

Table 2
CYP19 genotypes in bilateral breast cancer patients, monolateral breast cancer patients, healthy middle-aged females, and tumour-free elderly women

CYP19 genotypes	biBC	BC	HW	EW
$\Delta3(TTTA)_7/\Delta3(TTTA)_7$	3 (4%)	26 (9%)	13 (11%)	14 (15%)
$\Delta3(TTTA)_7/(TTTA)_7$	9 (13%)	29 (10%)	13 (11%)	6 (6%)
$\Delta3(TTTA)_7/(TTTA)_8$	6 (8%)	14 (5%)	1 (1%)	9 (9%)
$\Delta3(TTTA)_7/(TTTA)_{10}$	0	3 (1%)	1 (1%)	5 (5%)
$\Delta3(TTTA)_7/(TTTA)_{11}$	13 (18%)	58 (20%)	20 (17%)	15 (16%)
$\Delta3(TTTA)_7/(TTTA)_{12}$	2 (3%)	7 (2%)	1 (1%)	1 (1%)
$(TTTA)_7/(TTTA)_7$	4 (6%)	6 (2%)	10 (9%)	1 (1%)
$(TTTA)_7/(TTTA)_8$	2 (3%)	17 (6%)	9 (8%)	6 (6%)
$(TTTA)_7/(TTTA)_{10}$	0	1 (<0.5%)	0	1 (1%)
$(TTTA)_7/(TTTA)_{11}$	9 (13%)	30 (10%)	16 (14%)	6 (6%)
$(TTTA)_7/(TTTA)_{12}$	0	4 (1%)	2 (2%)	2 (2%)
$(TTTA)_8/(TTTA)_8$	3 (4%)	12 (4%)	4 (3%)	1 (1%)
$(TTTA)_8/(TTTA)_{10}$	0	4 (1%)	1 (1%)	1 (1%)
$(TTTA)_8/(TTTA)_{11}$	12 (17%)	27 (9%)	12 (10%)	13 (14%)
$(TTTA)_8/(TTTA)_{12}$	0	7 (2%)	0	0
$(TTTA)_9/(TTTA)_{10}$	0	0	0	1 (1%)
$(TTTA)_9/(TTTA)_{11}$	0	1 (<0.5%)	0	0
$(TTTA)_{10}/(TTTA)_{11}$	2 (3%)	3 (1%)	0	0
$(TTTA)_{10}/(TTTA)_{12}$	0	2 (1%)	0	0
$(TTTA)_{11}/(TTTA)_{11}$	5 (7%)	33 (11%)	11 (9%)	10 (10%)
$(TTTA)_{11}/(TTTA)_{12}$	1 (1%)	11 (4%)	3 (3%)	4 (4%)
Total genotypes	71 (100%)	295 (100%)	117 (100%)	96 (100%)

Table 3
Distinct prevalence of *CYP19* $\Delta3(TTTA)_7$ allele in premenopausal *versus* postmenopausal breast cancer patients

Clinical parameters[a]	$\Delta3(TTTA)_7$	$(TTTA)_7$	$(TTTA)_8$	$(TTTA)_9$	$(TTTA)_{10}$	$(TTTA)_{11}$	$(TTTA)_{12}$	Total alleles
BC patients								
Menopausal status								
Premenopausal ($n = 86$)	65 (38%)	18 (10%)	19 (11%)	1 (1%)	4 (2%)	61 (35%)	4 (2%)	172 (100%)
Postmenopausal ($n = 155$)	67 (22%)	60 (19%)	56 (18%)	0	5 (2%)	101 (32%)	21 (7%)	310 (100%)
Perimenopausal ($n = 17$)	9 (26%)	6 (18%)	7 (21%)	0	2 (6%)	9 (26%)	1 (3%)	34 (100%)
Age at onset (years)								
< 50 ($n = 94$)	72 (38%)	23 (12%)	20 (11%)	1 (1%)	7 (4%)	61 (32%)	4 (2%)	188 (100%)
> 50 ($n = 193$)	86 (22%)	69 (18%)	67 (17%)	0	6 (2%)	132 (34%)	26 (7%)	386 (100%)
biBC patients								
Menopausal status								
Both premenopausal ($n = 23$)	16 (35%)	9 (20%)	10 (22%)	0	1 (2%)	10 (22%)	0	46 (100%)
Both postmenopausal ($n = 25$)	8 (16%)	12 (24%)	9 (18%)	0	1 (2%)	18 (36%)	2 (4%)	50 (100%)
Pre- / post- ($n = 16$)	7 (22%)	7 (22%)	4 (13%)	0	0	14 (44%)	0	32 (100%)
Age at onset (years)								
Both $\leqslant 50$ ($n = 27$)	18 (33%)	10 (19%)	11 (20%)	0	1 (2%)	14 (26%)	0	54 (100%)
Both > 50 ($n = 26$)	9 (17%)	13 (25%)	10 (19%)	0	1 (2%)	19 (37%)	0	52 (100%)
$\leqslant 50$ / > 50 ($n = 12$)	5 (21%)	5 (21%)	2 (8%)	0	0	10 (42%)	2 (8%)	24 (100%)

[a] Data was missing for some patients.

4. Discussion

The present study describes the analysis of *CYP19* intron 4 polymorphisms in groups with distinct BC susceptibility and tolerance, namely biBC patients, BC patients, healthy middle-aged female donors and geriatric tumour-free women. Our data indicate the neutral role of the (TTTA)n polymorphism in BC predisposition, but show the association between the $\Delta3(TTTA)_7$ allele and menopausal status in both BC and biBC cohorts.

Several studies have analysed the (TTTA)n polymorphism in BC patients. Previous reports have described a predisposing role for distinct *CYP19* alleles, such as $(TTTA)_{12}$ [14,15], $TTTA_{10}$ [15–17], $(TTTA)_7$ [19], $(TTTA)_8$ [17], or alleles containing 10 or more TTTA repeats [24]. In the present study, we failed to observe any statistical difference in the (TTTA)n polymorphism between the affected and non-affected women. Our approach appears to have some advantages, as it includes groups with extreme characteristics of BC risk (biBC patients) and tumour resistance (geriatric controls). If any of the (TTTA)n alleles contributed to BC predisposition, we would expect to observe a gradual deviation of the allele frequency from biBC to BC to HW to EW categories. However, none of the data regarding the (TTTA)n variants fitted this association hypothesis.

Only a few reports have assessed the involvement of the 3-bp deletion/insertion *CYP19* variants in BC risk [16–19]. Moreover, none of these studies commented on the clinical data of BC patients in relation to the $\Delta3(TTTA)_7$ allele distribution. In our cohort of BC cases, we observed a statistically significant difference in the $\Delta3(TTTA)_7$ allele frequency between pre- and postmenopausal patients (38% versus 22%; $P=0.0001$; OR = 2.20 (95% CI 1.46–3.32)). Given the overall number of statistical comparisons made, this association itself may not be considered sufficient for conclusions to be drawn. However, the non-random association between the $\Delta3(TTTA)_7$ allele and BC menopausal status was strikingly supported by a similar tendency in the biBC group. Indeed, comparison of pre- and postmenopausal biBC cases produced an even higher OR, although the small number of observations led to a less significant P value (35% versus 16%; $P=0.035$; OR = 2.80 (1.08–7.23)). Therefore, when the distribution of *CYP19* polymorphism in the BC and biBC patients was analysed by the Mantel–Haenszel method for grouped data, the association between the $\Delta3(TTTA)_7$ allele and menopausal status produced very convincing statistical estimations ($P=0.000014$; OR = 2.29 (1.57–3.34)). The P value remained well within the level of significance after the Bonferroni correction was used for multiple comparisons (P corrected for the number of alleles ($n=7$): 0.000098; P corrected for the number of independent clinical parameters tested (menopausal status/age; tumour size; nodal involvement; family history ($n=4$): 0.000056; P corrected for both ($n=28$): 0.000392). Of note, overrepresentation of the $\Delta3(TTTA)_7$ allele in premenopausal breast cancer patients was accompanied by a corresponding deficit of several of the remaining alleles, that formally approached statistical significance in the BC group ($(TTTA)_7$: $P=0.011$; $(TTTA)_8$: $P=0.042$; $(TTTA)_{12}$: $P=0.035$)). However, these deviations were neither statistically confirmed in the biBC group, nor did they remained valid after the Bonferroni correction.

Thus, the $\Delta3(TTTA)_7$ allele appeared to increase the risk of premenopausal BC, but protected against postmenopausal disease. Distinct age-specific roles for the polymorphism of the key participant of steroidogenesis are somewhat expected from physiological assumptions. While in premenopausal women the main source of oestrogens is the ovary, peripheral conversion of androgens to oestrogens in adipose, muscle, bone and other tissues has a major impact in postmenopausal females [25]. Moreover, there is some evidence that not only the source, but the BC predisposing significance of steroid hormones may be different in pre- and postmenopausal women. For instance, several investigators have provided data for a protective role of a high oestrogen level before menopause, whereas in postmenopausal subjects increased concentration of oestrogens was associated with an elevated BC risk [26,27]. Taken together, the above mentioned observations fit the hypothesis of an association between the $\Delta3(TTTA)_7$ allele and relative oestrogen deficiency. However, the supportive endocrinological arguments should be treated with caution, as the functional relevance of the *CYP19* deletion/insertion polymorphism is unclear.

In conclusion, we have employed here a non-traditional design of a molecular epidemiological study, where the comparison of BC cases and healthy donors was supplemented by an analysis of subjects with extreme BC risk (biBC patients) or tolerance (tumour-free elderly women). Failure to find *CYP19* (TTTA)n allele deviations among these four groups strongly argues against the role of this tetranucleotide polymorphism in BC predisposition. However, we report for the first time the involvement of a 3 bp deletion/insertion *CYP19* polymorphism in BC development, that is exemplified by the overrepresentation of the $\Delta3(TTTA)_7$ allele in the premenopausal BC group, but its underrepresentation among postmenopausal cases. This association is very likely to be truly non-random, as significant differences between pre- and postmenopausal patients were observed for both BC and biBC patients. Further studies should clarify whether the effect of the $\Delta3(TTTA)_7$ variant is attributed to transcriptional regulation, or to linkage disequilibrium with other relevant polymorphisms.

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