Meta Gene 28 (2021) 100889

Contents lists available at ScienceDirect

Meta Gene

journal homepage: www.elsevier.com/locate/mgene

Thymidylate synthase and methylenetetrahydrofolate reductase polymorphisms and breast cancer susceptibility in a Brazilian population

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ARTICLE INFO

Keywords:
Breast cancer
Folate pathway
Susceptibility

ABSTRACT

Breast cancer (BC) is the most diagnosed cancer and the leading cause of cancer death in women worldwide. Polymorphisms in genes involved in the folate pathway are suggested as possible BC etiological factors. Among them, Thymidylate synthase (TYMS) has two polymorphisms related to BC: TSER and 1494del6. Methylenetetrahydrofolate reductase (MTHFR) has two SNPs, C677T and A1298C, that also could increase breast cancer risk. This work aimed to determine if TYMS and MTHFR genes polymorphisms are related to BC risk in a population of North Brazil. A total of 124 DNA samples, obtained from BC patients and control women, were genotyped utilizing PCR and automatic sequencing. Chi-square, G-test, Fisher's exact test, and odds ratio were applied to analyse the data. Significant associations with BC risk were observed for TYMS 3R allele carriers. Besides, patients with the −6 allele of 1494del6 polymorphism were more likely to develop aggressive BC molecular tumor types (Luminal B, HER2-positive, and Triple-Negative). In conclusion, TYMS polymorphisms are related to BC risk in the studied population, as the 3R allele is associated with BC susceptibility and the presence of the 1494del6–6 allele increases the development risk of more aggressive BC subtypes. Those findings may be useful for predicting the efficacy of anti-TS drugs in BC patients.

1. Introduction

Breast cancer (BC) is the most diagnosed cancer and the leading cause of cancer death in women across the world. In 2020, BC surpassed lung cancer as the most diagnosed cancer worldwide with more than two million new cases registered (Fahad Ullah, 2019; Sung et al., 2021).

There are numerous risk factors such as sex, aging, estrogen, family history, gene mutations, and unhealthy lifestyle, which can increase the possibility of developing BC (Sun et al., 2017). Epigenetic modifications in the tumor microenvironment as well as folate deficiency can also increase BC risk. Biological functions of folate within so-called 'one-carbon metabolism' are to facilitate de novo dNTP synthesis and to provide methyl groups required for intracellular methylation reactions (da Silva Nogueira et al., 2012; Sun et al., 2017; Suzuki et al., 2008). It is known that polymorphisms in genes encoding critical enzymes involved in the folate pathway play an important role in folate metabolism influencing the risk of cancer, being described as possible etiological factors of BC as well as an important factor in the response and outcome of chemotherapy (da Silva Nogueira et al., 2012).

Thymidylate synthase gene (TYMS), located at 18p11.32, encodes the thymidylate synthase enzyme (TS) that catalyzes the deoxyuridine monophosphate (dUMP) conversion to deoxythymidine monophosphate (dTMP), essential for thymidine production for DNA repair and synthesis (da Silva Nogueira et al., 2012). Due to the critical role of TS in nucleotide metabolism, this gene is a target of several chemotherapeutic agents including 5-fluorouracil (5-FU), capecitabine, and pemetrexed (Mandola et al., 2003). TYMS has polymorphisms in the 5′- and 3′- untranslated regions (UTRs), that influence gene expression (Yim et al., 2010). TSER (rs34743033) is a polymorphic 28 base-pair tandem repeats, resulting in two common alleles with double (2R) and triple (3R) repeats, uncovered in the 5'-UTR of the TYMS enhancer region and have been shown to influence gene expression. Another polymorphism in TYMS is 1494del6 (rs34489327) consisting of a 6 bp deletion of the sequence TTAAAG at nucleotide 1494 of the TYMS mRNA. Little is

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https://doi.org/10.1016/j.mgene.2021.100889
Received 7 December 2020; Received in revised form 21 February 2021; Accepted 14 March 2021
Available online 17 March 2021

currently known about the 3′-UTR of TYMS, but 3′-UTRs have been implicated in the modulation of gene regulation at the post-transcriptional level in many mammalian systems (Mandola et al., 2003).

Methylenetetrahydrofolate reductase gene (MTHFR) located at 1p16.22, encodes the cytoplasmic flavoenzyme MTHFR, which catalyzes the conversion of 5,10-MTHFR to 5-MTHFR, providing a source of methyl groups for biological reactions (Castro et al., 2004). There are two polymorphisms in MTHFR, C677T (rs1801133) and A1298C (rs1801131), that reduce enzymatic activity by as much as 30% for the 677T and 1298C alleles (Castro et al., 2004). The C677T SNP results in the substitution of alanine with valine at codon 222. This substitution generates a thermolabile enzyme and is associated with high homocysteine concentrations, especially in those individuals with low folate levels (Castro et al., 2004; Henríquez-Hernández et al., 2010; Rezende et al., 2017). The A1298C SNP results in the substitution of glutamate for an alanine at position 429 of the enzyme, where it may be involved in protein stabilization and resulting in decreased enzyme activity (van der Put et al., 1998; Weisberg et al., 1998).

Considering the metabolic role of TYMS and MTHFR genes in the development of various diseases, including BC (de Carvalho Barbosa et al., 2012; da Silva Nogueira et al., 2012; Gonzales et al., 2017), the purpose of this study was to investigate the association of these polymorphisms with BC risk in a population of Para State, North Brazil.

2. Material and methods

2.1. Samples collection and processing

A total of 124 individuals, divided into two distinct groups (61 cases and 63 controls) were enrolled in this research. Control blood samples were obtained from the Clinical Analysis Laboratory of the Federal University of Pará, from selected women without a history of neoplasia. Carcinoma samples (cases) were obtained from patients who underwent surgical treatment after BC diagnosis at the Ophir Loyola Hospital, in Belém, Brazil. All selected samples were of invasive ductal carcinoma locally advanced, stage III (A or B) according to the TNM scale (Sobin et al., 2011). BC samples were subjected to histopathological analysis with hematoxylin-eosin and immunohistochemistry and classified into four corresponding molecular subtypes: luminal A, luminal B, HER2-positive non-luminal, and Triple-Negative BC or TNBC (Vuong et al., 2014). All patients signed an informed consent term and the project was approved by the Ethics Committee of the Ophir Loyola Hospital" (Protocol n° 184.445/2013).

2.2. DNA extraction and polymorphisms genotyping

Genomic DNA extraction was conducted using the QIAamp DNA Mini kit (Qiagen) following the manufacturer's recommendations. For TSER polymorphism the primers and conditions described by (Ulrich et al., 2002) were used. For 1494del6 polymorphism the Forward 5′-TTCCCTCAAATCTGAGGGAGCTG-3′ and Reverse 5′-CTGCTCAGTTCCTTCCTAAAATA-3′ primers, designed by the authors, with an annealing temperature of 62 °C and 35 cycles were used. For MTHFR polymorphisms, primers and PCR conditions were previously described (Araújo et al., 2015; Yousef et al., 2018). TSER, C677T, and A1298C polymorphic bands were confirmed after 2% agarose gel electrophoresis. 1494del6 polymorphic bands were confirmed after 16% non-denaturing acrylamide gel electrophoresis. All the fragments obtained were purified using the EZ-10 Spin Column PCR Product Purification kit (Bio Basic) following manufacturer instructions and sequenced using an ABI3130 automatic sequencer (Life Technologies). Sequences were aligned using the BioEdit v7.0.5 software (Hall, 1999).

Table 1
Epidemiological data and characteristics of the patients.

Epidemiological data and patients' characteristics		Cases n (%)	*p*-value
Age	<50	36 (59.02)	<0.0001[a]
	≥50	25 (40.98)	
	Media	47.80 ± 11.04	
Race	Caucasian	18 (29.51)	<0.0001[a]
	Afro-Americans	12 (19.67)	
	Mixed race	31 (50.82)	
Body Mass Index (BMI)	≤24,9	18 (29.51)	<0.0001[a]
	25–29,9	24 (39.34)	
	30–34,9	16 (26.23)	
	≥35	3 (4.92)	
Menarche	≤12 years old	22 (30.07)	0.587
	>12 years old	39 (63.93)	
Pregnancy	No	5 (8.20)	< 0.0001[a]
	Yes	56 (91.80)	
Menopause	Yes	29 (47.54)	0.587
	No	32 (52.46)	
BC family history	Yes	20 (32.79)	< 0.0001[a]
	No	41 (67.21)	
Smoking	Yes	10 (16.39)	< 0.0001[a]
	No	51 (83.61)	
Alcohol consumption	Yes	4 (6.56)	< 0.0001[a]
	No	57 (93.44)	

[a] Statistically significant.

2.3. Statistical analysis

Associations between polymorphisms, clinical parameters, and BC risk were verified in GraphPad Prism version 5.01 (www.graphpad.com) and R Studio version 1.2.5001 (www.rstudio.com) using Chi-square (X2), G-test, Fisher's exact test, and Odds Ratio (OR) with 95% confidence interval (95%CI). All the results were considered significant when $p \leq 0.05$.

3. Results and discussion

The median age of the cases and controls was 47.80 ± 11.04 and 47.73 ± 13.15 years old, respectively ($p = 0.818$). Data about reproductive and BC family history and lifestyle such as smoking status and alcohol consumption of the patients are shown in Table 1.

The results of clinical data showed most patients were under 50 years old at the diagnosis. It is known that BC incidence and mortality increase proportionally with age (Winters et al., 2017) however, these results agree with the literature as in Brazil, there is a higher BC prevalence in younger women (Dos-Santos-Silva et al., 2019; Orlandini et al., 2021), especially at advanced stages (de Oliveira et al., 2021) than in other populations. Besides, it should be pointed out that 52.8% (19/36) of all patients under 50 years old have BC family history (OR = 26.82; 95%CI = 3.27–220.08; $p = 0.0002$), suggesting family history is a strong risk factor in the analyzed population as stated elsewhere (Rojas and Stuckey, 2016; Sun et al., 2017).

Another interesting feature of the studied population is that most patients had, at least, one pregnancy, all of them at an early age (under 30 years old) which disagrees with the literature (Sun et al., 2017; Winters et al., 2017). However, no correlation between parity and other clinical data such as age at menarche, lactation, abortion, and family history was observed that could explain these results.

Although smoking and alcohol consumption status are considered risk factors for BC (da Silva Nogueira et al., 2012), in this study overweight was the only extrinsic risk factor observed in the analyzed population ($p \leq 0.0001$). However, despite significant differences were observed in alcohol and smoking status, which might suggest the lack of consumption as BC risk factors, these results should be carefully interpreted as eventual smoking and alcohol consumption might not be admitted by the patients, resulting in a misinterpretation of the questionnaire.

Table 2
Genotypic and allelic frequencies distribution of analyzed polymorphisms.

Gene/ Polymorphism	Genotypic frequency				Allelic frequency		
	Genotype	Cases n (%)	Controls n (%)	*p* value	Allele	Patients	Controls
TYM	2R/2R	8 (13.3)	18 (28.5)	0.004[a]	2R	0.36	0.44
TSER	2R/3R	27 (45.0)	35 (55.6)		3R	0.64	0.56
	3R/3R	25 (41.7)	10 (15.9)				
TYM	+6/+6	26 (42.6)	16 (25.4)	0.13	+6	0.62	0.52
1494del6	+6/−6	24 (39.3)	33 (52.4)		−6	0.38	0.48
	−6/−6	11 (18.0)	14 (22.2)				
MTHFR	C/C	31 (50.8)	32 (50.8)	0.75	C	0.70	0.72
C677T	C/T	24 (39.3)	27 (42.9)		T	0.30	0.28
	T/T	6 (9.8)	4 (6.3)				
MTHFR	A/A	25 (41.0)	7 (43.8)	0.49	A	0.68	0.72
A1298C[b]	A/C	33 (54.1)	9 (56.2)		C	0.32	0.28
	C/C	3 (4.9)	0 (0)				

[a] Statistically significant.
[b] Small number of control samples due to DNA depletion.

Regarding the molecular markers, all genotypic and allelic frequencies were in Hardy-Weinberg equilibrium in both groups (cases and controls) (Table 2). It is worth noting that regarding TSER polymorphism, an individual with a rare allele (3R/4R) was observed in the cases group, which may be due to the contribution of the African continent in Brazil and the process of miscegenation, since the 4R allele has been reported, with high frequencies, in African populations (Marsh et al., 2000). However, as this case was considered an outlier, it was excluded from statistical analysis.

No differences between cases and controls were observed in all analyzed polymorphisms, but for TSER (Table 2). It is worth mentioning that although the observed TSER allelic frequencies were higher than those previously described for other Brazilian (de Carvalho Barbosa et al., 2012; da Silva Nogueira et al., 2012) and Latin American populations (Vázquez et al., 2017), they were similar to those described for Iranian (Rahimi et al., 2021) and Chinese (Zhai et al., 2006) populations. Furthermore, it is worth noting that a significant difference at TSER allelic and genotypes distributions ($p = 0.004$, Table 2) under all tested models of inheritance (Table 3) was observed between cases and controls, suggesting that the presence of the 3R allele is a risk factor for BC, similar as previously described (Rahimi et al., 2021).

The absence of correlation between C677T and A1298C polymorphisms and BC risk was also reported in an Italian population (Castiglia et al., 2019). On the other hand, as C677T and A1298C variants seem to be a BC predisposal factor in Asians (He and Shen, 2017; Rezaee et al., 2021), those inconsistent findings could be due to different ethnic groups, environmental factors, and complex epigenetic pathways that lead to carcinogenesis, and thus the association of these polymorphisms with BC is still controversial (Gallegos-Arreola et al., 2008; Hedayatizadeh-Omran et al., 2017; Henríquez-Hernández et al., 2010; Hesari et al., 2018; Kumar et al., 2010; Rahimi et al., 2019; Villegas et al., 2018; Wang et al., 2011; Waseem et al., 2016).

Regarding the molecular classification, most of the samples were of Luminal B subtype (28/61, 45.9%) followed by TNBC (17/61, 27.9%), HER2-positive (9/61, 14.8%), and Luminal A (7/61, 11.4%). It is worth mentioning that in the analyzed population, Luminal B was the most frequent BC molecular subtype, differing from the observed in other Brazilian populations, where the most frequent molecular subtype is Luminal A, followed by Luminal B, TNBC, and HER2-positive (Brincas et al., 2020; Simon et al., 2019). However, it is noteworthy that a previous study in the same population had similar results, although with different frequencies (dos Silva et al., 2020), suggesting this distribution must be due to a regional characteristic. Besides, it should also be because all samples used were at advanced stages (stage III), which could also explain the distribution of the subtypes.

Concerning molecular subtypes, statistically significant differences were observed only at 1496del6 alleles, where the presence of the −6 allele was associated with more aggressive molecular subtypes (Luminal B, HER2-positive and TNBC) (OR = 10.2000, $p = 0.04$, IC95% = 1.1439–90.9486), as the presence of the +6 allele under the dominance model confers a protective effect (Table 4). Despite no statistical difference was observed between cases and controls regarding 1494del6 polymorphism as previously related in other reports (Henríquez-Hernández et al., 2010), as far as we know, this is the first study in a Brazilian population showing that the presence of the −6 allele is associated with more aggressive molecular BC subtypes (Luminal B, HER2-positive and TNBC). This could be related to the fact that 1494del6 polymorphism may affect the level of *TYMS* mRNA (Kumar et al., 2010), and in the stability of TYMS protein (Gallegos-Arreola et al., 2008; Wang et al., 2011). It should be noticed that TS levels can be significantly different among BC subtypes and can be useful to improve treatment strategies, as its expression could be clinically important for predicting the efficacy of anti-TS drugs (Shan et al., 2018; Siddiqui et al., 2019).

TYMS gene expression is a well-established chemotherapy target, as its overexpression represents a major resistance mechanism to 5-fluorouracil (5-FU) (Chao and Anders, 2018; González-Neira, 2012), and pemetrexed chemotherapy (Shan et al., 2018), indicating that these polymorphisms may be useful as diagnostic and prognostic markers for BC, being helpful in chemotherapy regimens decision (Shan et al., 2018). Besides, several studies also suggest the use of *TYMS* as a potential biomarker as higher enzyme levels are strongly correlated with worse prognosis, especially in the most aggressive subtypes of BC (Kakimoto et al., 2005; Shan et al., 2018; Siddiqui et al., 2019; Song et al., 2021).

4. Conclusion

The analyzed data suggest, for the first time in the studied population, the association of TSER polymorphism with BC risk. Besides, an association between the presence of TS 1496del6 allele with an increased risk of developing more aggressive molecular BC subtypes (Luminal B, HER2-positive, and TNBC) is also observed which may be useful for predicting the efficacy of anti-TS drugs and to determine a targeted therapy in these BC subtypes.

Funding

This research was supported by funds from the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNP; 431801/2016-9 and 311544/2018-5 to BNB, 301350/2019-1 to RMRB). MACD was awarded with a master fellowship from the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES).

Table 3
Genotype distributions and corresponding risk assessments for BC using genetic models of inheritance.

Polymorphism/ Model	Genotype	Cases n (%)	Controls n (%)	OR IC 95%	*p*
TSER					
Codominant	3R/3R	25 (41.7)	10 (15.9)	1.00 (Reference)	–
	2R/3R	27 (45.0)	35 (55.6)	3.24 (1.33–7.08)	0.015[a]
	2R/2R	8 (13.3)	18 (28.5)	5.63 (1.85–18.06)	0.004[a]
Dominant	3R/3R	25 (41.7)	10 (15.9)	1.00 (Reference)	
	2R/3R + 2R/2R	35 (58.3)	53 (84.1)	3.79 (1.62–8.85)	0.003[a]
Recessive	3R/3R + 2R/3R	52 (86.7)	45 (71.5)	1.00 (Reference)	–
	2R/2R	8 (13.3)	18 (28.5)	2.60 (1.03–6.55)	0.048[a]
1494del6					
Codominant	+6/+6	26 (42.6)	16 (25.4)	1.00 (Reference)	–
	−6/+6	24 (39.3)	33 (52.4)	2.23 (0.99–5.05)	0.08
	−6/−6	11 (18.0)	14 (22.2)	2.07 (0.76–5.65)	0.24
Dominant	+6/+6	26 (42.6)	16 (25.4)	1.00 (Reference)	–
	−6/+6 + −6/−6	35 (57.4)	47 (74.6)	2.18 (1.02–4.67)	0.066
Recessive	+6/+6 + −6/−6	50 (82.0)	49 (77.8)	1.00 (Reference)	–
	−6/−6	11 (18.0)	14 (22.2)	1.30 (0.54–3.14)	0.72
C677T					
Codominant	C/C	31 (50.8)	32 (50.8)	1.00 (Reference)	–
	C/T	24 (39.3)	27 (42.9)	1.09 (0.52–2.28)	0.97
	T/T	6 (9.8)	4 (6.3)	0.65 (0.17–2.51)	0.77
Dominant	C/C	31 (50.8)	32 (50.8)	1.00 (Reference)	–
	C/T + T/T	30 (49.2)	31 (49.2)	1.00 (0.50–2.02)	0.86
Recessive	C/C + C/T	55 (90.2)	59 (93.7)	1.00 (Reference)	–
	T/T	6 (9.8)	4 (6.3)	0.62 (0.17–2.32)	0.70
A1298C[b]					
Codominant	A/A	25 (41.0)	7 (43.8)	1.00 (Reference)	–
	A/C	33 (54.1)	9 (56.2)	0.32 (0.08–1.38)	0.22
	C/C	3 (4.9)	0 (0)	–	–
Dominant	A/A	25 (41.0)	7 (43.8)	1.00 (Reference)	–
	A/C + C/C	36 (59.0)	9 (56.2)	0.89 (0.29–2.71)	0.93
Recessive	A/A + A/C	58 (95.1)	16 (100)	1.00 (Reference)	–
	C/C	3 (4.9)	0 (0)	–	–

[a] Statistically significant.
[b] Small number of control samples due to DNA depletion.

Table 4
Genotype distributions and corresponding risk assessments for the most aggressive BC molecular subtypes using genetic models of inheritance.

Polymorphism/ Model	Genotype	Luminal B+ HER2-positive+ TNBC n (%)	Luminal A n (%)	OR IC 95%	p
TSER					
Codominant	3R/3R	6 (11.3)	2 (28.6)	1.00 (Reference)	–
	2R/3R	24 (45.3)	3 (42.8)	0.38 (0.05–2.77)	0.68
	2R/2R	23 (43.4)	2 (28.6)	0.26 (0.03–2.25)	0.51
Dominant	3R/3R	6 (11.3)	2 (28.6)	1.00 (Reference)	–
	2R/3R + 2R/2R	47 (88.7)	5 (71.4)	0.31 (0.05–2.02)	0.50
Recessive	3R/3R + 2R/3R	30 (56.6)	5 (71.4)	1.00 (Reference)	–
	2R/2R	23 (43.4)	2 (28.6)	0.52 (0.09–2.94)	0.73
1494del6					
Codominant	+6/+6	20 (37.0)	6 (85.7)	1.00 (Reference)	–
	−6/+6	23 (42.6)	1 (14.3)	0.15 (0.02–1.31)	0.13
	−6/−6	11 (20.4)	0 (0)	–	–
Dominant	+6/+6	20 (37.0)	6 (85.7)	1.00 (Reference)	–
	−6/+6 + −6/−6	34 (63.0)	1 (14.3)	0.10 (0.01–0.87)	0.04[a]
Recessive	+6/+6 + −6/−6	43 (79.6)	7 (100)	1.00 (Reference)	–
	−6/−6	11 (20.4)	0 (0)	–	–
C677T					
Codominant	C/C	29 (53.7)	2 (28.6)	1.00 (Reference)	–
	C/T	20 (37.0)	4 (57.1)	2.90 (0.48–17.38)	0.44
	T/T	5 (9.3)	1 (14.3)	2.90 (0.22–38.32)	0.98
Dominant	C/C	29 (53.7)	2 (28.6)	1.00 (Reference)	–
	C/T + T/T	25 (46.3)	5 (71.4)	2.90 (0.52–16.28)	0.40
Recessive	C/C + C/T	49 (90.7)	6 (85.7)	1.00 (Reference)	–
	T/T	5 (9.3)	1 (14.3)	1.63 (0.16–16.43)	0.80
A1298C[b]					
Codominant	A/A	22 (40.7)	3 (42.9)	1.00 (Reference)	–
	A/C	29 (53.7)	4 (57.1)	1.22 (0.25–6.08)	0.87
	C/C	3 (5.6)	0 (0)	–	–
Dominant	A/A	22 (40.7)	3 (42.9)	1.00 (Reference)	–
	A/C + C/C	32 (59.3)	4 (57.1)	0.92 (0.19–4.51)	0.76
Recessive	A/A + A/C	51 (94.4)	7 (100)	1.00 (Reference)	–
	C/C	3 (5.6)	0 (0)	–	–

[a] Statistically significant.
[b] Small number of control samples due to DNA depletion.

Contributors

MACD and BNB conceived the study. MACD, MDA and DRP processed the samples, conducted the experiments and the statistical analysis. MDA, DRP and RMRB collected the samples and clinical data. MACD and BNB wrote the manuscript. BNB, DRP and RMRB reviewed the paper. All authors read and approved the final manuscript.

Conflict of interest statement

The authors whose names are listed immediately below certify that they have NO affiliations with or involvement in any organization or entity with any financial interest (such as honoraria; educational grants; participation in speakers' bureaus; membership, employment, consultancies, stock ownership, or other equity interest; and expert testimony or patent-licensing arrangements), or non-financial interest (such as

personal or professional relationships, affiliations, knowledge or beliefs) in the subject matter or materials discussed in this manuscript.

Declaration of Competing Interest

The authors declare no conflict of interest in this manuscript.

Acknowledgements

The authors would like to thank the Ophir Loyola Hospital, Universidade Federal do Pará and the volunteers (patients and control) that participated in this study.

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