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Genetic Diversity and Structuring of the BRAF gene in Breast Tumors

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Abstract

The International Agency for Research on Cancer (IARC) estimates an increase in breast cancer worldwide. In Senegal, the most common malignant tumour in women is breast cancer. But also in Senegal as in most African countries, benign tumours occupy an important place in mammary pathologies. To better understand the impact of nucleotide variability and genetic instability of benign and malignant breast tumours, we used the *BRAF* gene, which is a nuclear gene. This work allowed us to compare the polymorphism, diversity, structure and genetic evolution of exon 15 of the *BRAF* gene between patients with benign tumours, malignant breast tumours and control subjects. The analysis at the identification of mutation of exon 15 of the *BRAF* gene led us to conclude that *BR*AF was mutated in (1.5%) of cases. We observed a synonymous mutation A598A in malignant tumours. Our results showed that somatic mutations of this *BRAF* gene were common in Senegalese patients with both benign and malignant breast tumours. So our results allowed us to conclude that the *BRAF* gene is involved in breast tumours.

Keywords: cancer; breast; mutations; *BRAF*

Introduction

BRAF is a proto-oncogene involved in the KRAS MAP-kinase intracellular signaling pathway. The activation of membrane receptors by binding with their specific ligands induces a cascade activation of this pathway and the stimulation of several cellular functions. This occurs in many types of cells. *BRAF* is normally activated by RAS proteins. It can also be activated by mutations called "function gain" or "activators"; the proto-oncogene then becomes an oncogene. Activating mutations of the *BRAF* gene were first detected in human tumors about eighteen years ago [1], and their frequency varies greatly according to tumour type. Mutations generally appear during the early phases of oncogenesis. *BRAF* mutations are mainly located in the exon 15 activator segment, *i.e.* acquired, somatic and non-germinal mutations [2]. The absence of germline mutation can be explained by molecular genetic experiments in mice: *BRAF* mutations induce embryonic lethality [2]. The most frequent mutation is a localized punctually mutation at exon 15 (thymine [T] 1799 has been transformed into adenine [A] - gTg/gAg) substituting a valine (V) into glutamic acid at position 600 of the protein (V600E); this mutation is found in the vast majority of cancers with a mutated *BRAF* form [1]. The mechanisms of acquisition of the V600E mutation in the protein are probably linked to alternative mechanisms that have not yet been identified. The mutated protein *BRAF* V600E has 500 times more kinase activity than the wild form of *BRAF*, which stimulates ERK protein phosphorylation and cell signaling in a disproportionate way [1]. Apart from the V600E mutation, other somatic mutations of *BRAF* have been described in human melanomas:

In mutated melanomas for *BRAF*, 74 to 90% are V600E and 16 to 29% are V600K. Depending on the studies, the proportions of one type of mutation compared to another are slightly different. *BRAF*'s amino acid V600 is located in the kinase activation domain, near the threonine 599 and serine 602 residues on which phosphorylation induces kinase activity. The V600E mutation could thus simulate the phosphorylation of threonine 599 and serine 602. Another hypothesis of the uncontrolled activation mechanism is the increase in exposure of the activation segment when a small hydrophobic amino acid (valine) is replaced by a hydrophilic residue (glutamic acid), [3]. The *BRAF* gene is mutated in the majority of patients with melanoma and a minority of patients with breast, colon and lung cancer [4]. In this study, the hypothesis of the existence of nucleotide mutations involved in breast tumors in Senegalese women has been put forward. For this purpose, exon 15 of the *BRAF* gene, which is a nuclear gene, was chosen to test this hypothesis.

Methodology

Patients and samples

The study involves sixty-six (66) surgical samples composed of malignant and benign tumors and twelve (12) blood samples from patients who are managed at the Aristide Le Dantec Hospital Cancer Institute. Samples were collected from these patients after informed and written consent in a standardized form.

DNA extraction, amplification and sequencing

DNA extraction was performed from tissues using the Qiagen DNeasy kit. Quality of the extracted DNA was verified by electrophoretic migration in 1,5% of agarose gel; DNA was then stored at a temperature of 20°C. PCR amplification of exon 15 of *BRAF* gene was carried out at a reaction volume of 50 µL containing 1 µL of DNA and 49 µL of the PCR mix comprising 34.9µL of MilliQ water, 5 µL of buffer 10X, 2 µL of MgCl2, 4µL of dNTP, 1.5 µL of each primer (Forward 5'- TCATAATGCTTGCTCTGATAGGA -3') (Reverse 5'- GGCCAAAAATTTAATCAGTGGA -3'). The PCR program included the following conditions: 95°C for 3 min; 35 cycles (95°C for 30 s; 55°C for 30 s; 72°C 30 s); 72°C for 10 min. PCR products were purified and sequenced. Sequencing reactions were performed using an MJ Research PTC-225 Peltier thermocycler with the ABI PRISM kit and electrophoresed in an ABI 3730 XL sequencer.

Molecular analyses

The exon 15 sequences of the BRAF gene, from the three groups (malignant, benign and control), are carefully verified, corrected and aligned with Bio Edit software version 7.0.8 [5]. Alignment is indeed an important step in data analysis. It is used in particular to highlight the similarities between the sequences by finding the position of deletions, insertions, and probable substitutions.

To determine the position, nature and frequency of exon 15 mutations in the *BRAF* gene, the raw sequencing data were submitted to Mutation Surveyor version 5.0.1 (www.softgenetics.com). Indeed, this software compares the chromatograms submitted with the reference sequence of the gene of interest incorporated in the database of the said software but also with sequences from the Genbank database (https://www.ncbi.nlm.nih.gov/genbank/). Thus this software allows detecting the presence of heterozygous mutations (het), insertions (in) and deletions (del).

In addition to the universality of the DNA molecule in the living world, there is its variability. This variability results in random changes in the DNA sequence (mutations) that can affect the cellular activity and the entire body. Therefore, to estimate the genetic diversity of the *BRAF* gene, we determined the number of variable and invariable sites, the number of informative sites, the total number of mutations, the number of haplotypes (h), the average number of nucleotide differences (k), the haplotypic (HD) and nucleotide (π) diversity, using the DnaSP software version 5.10 [6]. Nucleotide frequencies, nature of mutations (% transitions and transversions) and molecular distances with the Kimura 2 Parameter (K2P) model were calculated with the MEGA program version 6.06 [7]. Nucleotide frequencies and molecular distances were also calculated at each codon position.

The nucleotide sequences of the *BRAF* gene are transformed into amino acid sequences using MEGA software version 6.06 [7], using the best reading frame. The level of significance of amino acid frequency variations between the three groups (controls, benign tumors, and malignant tumors) was demonstrated by the chi2 test with a level of significance (P-value) of0.05.

Genetic distances between controls vs TB, controls vs TM and TB vs TM at the intra- and inter-individual level were explained by Nei's genetic distance using the MEGA software version 6.06 [7].

We conducted demogenetic tests that compare the level of adjustment between diversity to the three groups and expected theoretical values. Among these tests: the D of Tajima [8], the D*and F* of Fu and Li [9] and the H of Fay and Wu [10] and the R2 of Ramos [11]. These different estimators are obtained with the DnaSP version 5.10 programs [6] and Harlequin version 3.5.1.3 [12]. By choosing as a starting hypothesis that exon 15 of the *BRAF* gene is under positive selection, the existence of any selection has been apprehended by a positivity (dN>dS) thanks to the MEGA 6 software with dN is the non- synonymous substitution rate and dS is the synonymous substitution rate. This test was performed using the Nei-Gojobori model and the pairwise deletion method. A value of $P < 0.05$ was considered significant with a bootstrap value of 10000 replications. After the demogenetic tests, we determined the analysis of the distribution disparity (Mismatch distribution), which is the graphical representation of the distribution of genetic distances existing between individuals. Mismatch's analysis is accompanied by two indices that test the quality of adjustment of the distribution. These indices are the SSD (sum of squares of deviations) and the Rag (irregularity index). The graphs are built with DnaSP software version 5.10 [6]. The SSD and Rag indices were obtained with Harlequin software version 3.5.1.3[12].

Results

A total of 86 sequences (12 for controls, 31 for benign tumors and 35 for malignant tumors) were sequenced, aligned and analyzed.

Nature and frequency of mutations

In benign tumors, we find the mutations L588H and D594V (1.5%), which correspond to a change of amino acid in position 588 in BRAF, with leucine (L) replaced by a histidine (H) and D594V to a change of amino acid in position 594 in BRAF with an aspartic acid (D) replaced by valine (V), Table 1. We also observe a synonymous mutation A598A, in malignant tumors.

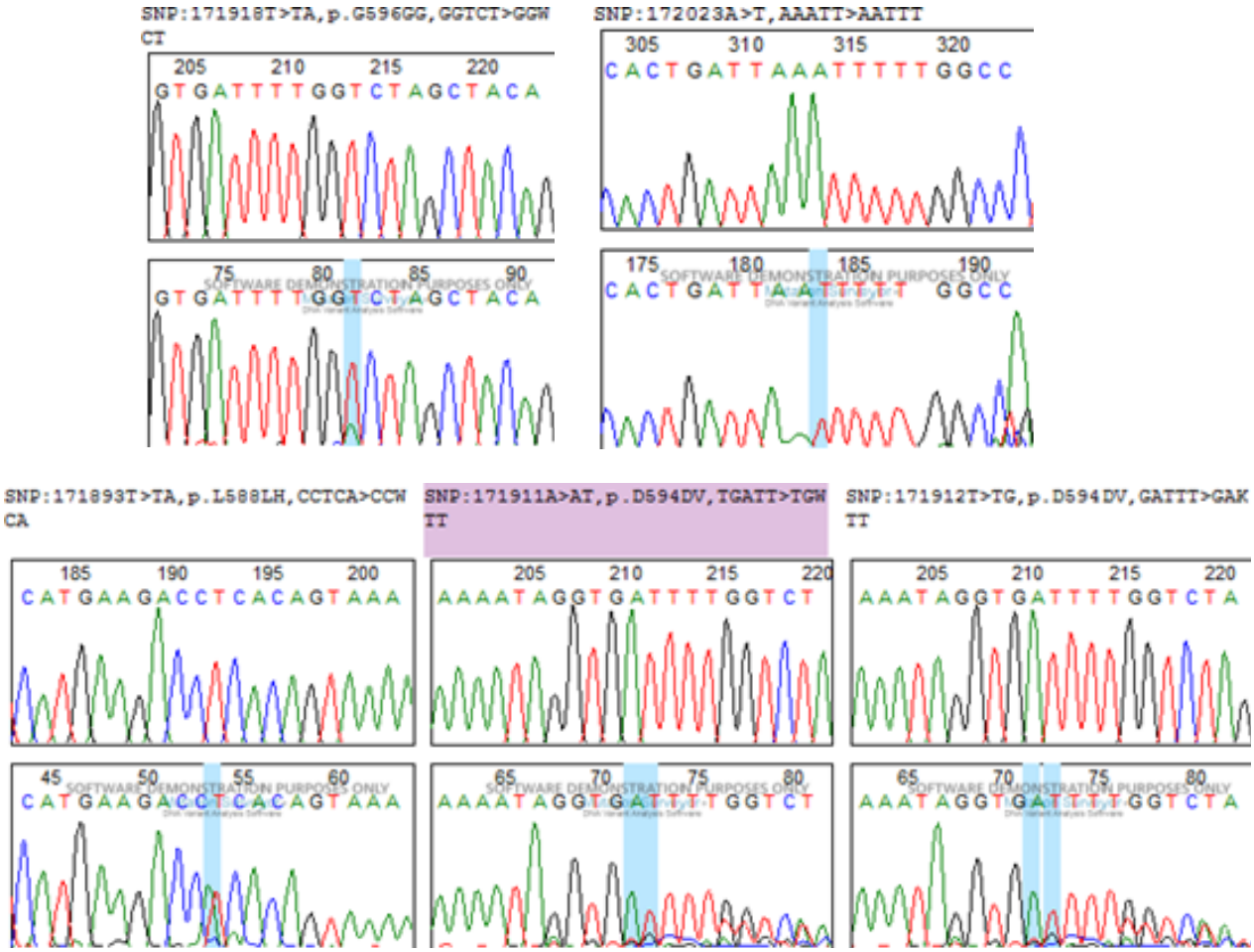
Genetic diversity

The analysis in Table 2 shows that the *BRAF* gene is more diverse in terms of malignant tumors and TB than in controls. However, we note a great diversity among TMs. This increased diversity in malignant tumors is reflected in a high number of variable sites (20) compared to TB (17) and controls (8). We also note a higher total number of mutations (26) for TMs compared to TB (22) and controls (10). The average number of nucleotide differences (k) is higher at the TM levels (3.615) compared to TB (3.009) and controls (2.045).We find that the percentages of transversions are higher than those of transitions in the three groups. However, we note that in the percentages of transversions they are higher in controls and benign tumors respectively (73.42%) and (78.82%) compared to the TM (53.16%). In contrast, in malignant tumors, transitions (46.82%) are higher compared to TB (21.18%) and controls (26.51%) (Table 2). Analysis of the diversity indices reveals high haplotypic diversity of (0.818), (0.920) and (0.934) respectively in malignant, benign and control tumors against low nucleotide diversity (0.01130), (0.01662) and (0.01997) (Table 2).

In the nucleotide frequency diagram, we see that bases A and T are the most dominant with respectively 27% and 35% for all three groups compared to bases C and G respectively 17.5% and 20%. And we also notice a predominance of (A +T) in all three groups with a percentage of 62% compared to (C+G) 38%.

NUCLEOTIDE POSITION	AMINO ACIDS	NUMBER
CONTROLS		
172023A>AT		2
BENIGN TUMORS		
171893T>TA ; mut het T1763A	L588H	1
171918T> TA ; mut het T1788A	G596G	1
171911A>AT ; mut het A1781T	D594V dbSNP 121913338	1
171912T>TC ; mut het T1782C	D594V	1
172023A>AT		3
172032C>CA		1
MALIGNANT TUMORS		
171924T>TA ; mut het T1794A	A598AA	1
172021A>AT		4
172020T>TA		1
172023A>AT		1
172032C>CA		1
172032C>CT		1

Table 1: Nature and frequency of mutations



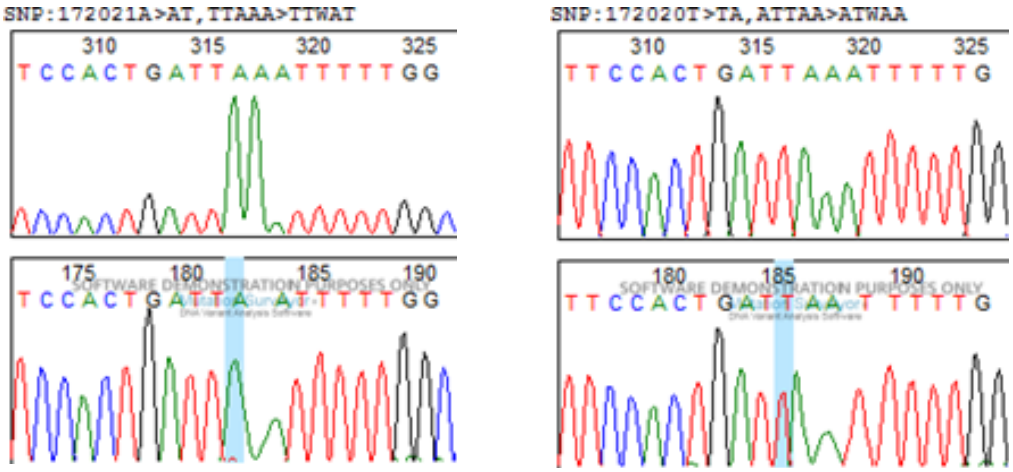


Figure 1: BRAF gene mutations

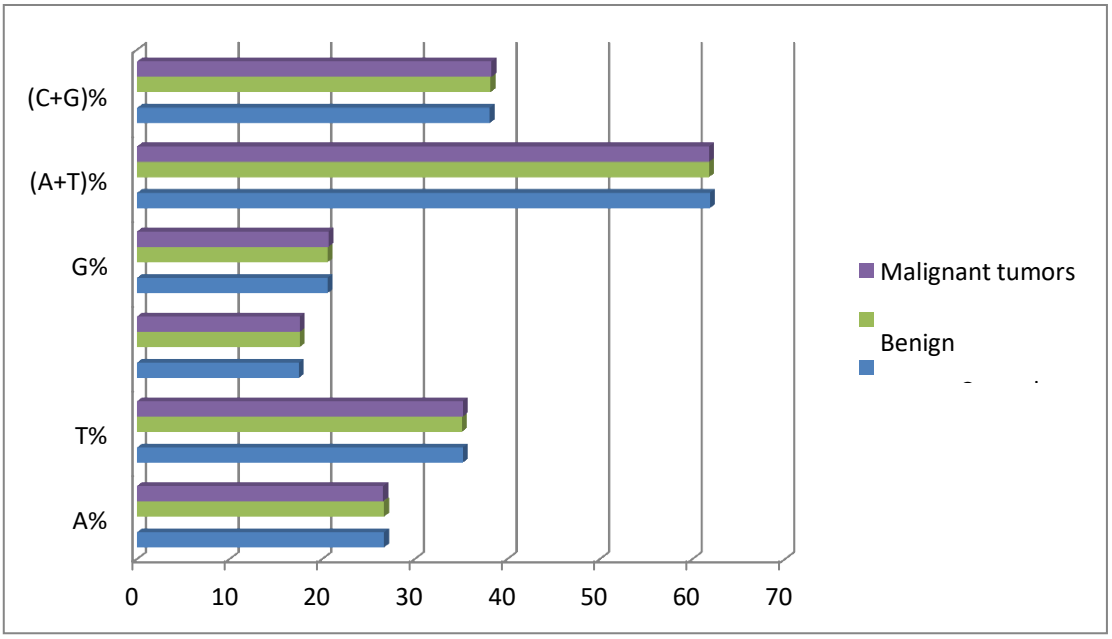


Figure 2: Nucleotide frequencies of the sequences

Variability of BRAF amino acids

The analysis of inter-tissue protein diversity (Table 3) by highlighting amino acid variations indicates that, despite slight variations in amino acid frequencies between the three groups, no significant difference could be determined.

Genetic differentiation

The genetic distance values (d) at the intra and inter tissue level and the degree of genetic differentiation (Fst) between controls and benign tumors and between controls and malignant tumors as well as benign and malignant tumors are recorded in Table 4. The analysis of genetic distances between controls and malignant tumors and benign and malignant tumors revealed a low respective genetic diversity (d=0.0159), (0.0186), but greater than that between controls and benign tumors (d=0.0139). We note that within malignant and benign tumors the values of the genetic distance are equal (d=0.02), higher than those of the controls (d=0.01). We also find that the degree of genetic differentiation shows an Fst that is equal to 0 and not significant (0; P-value=0.86486), (0; P-value=0.76577) and (0; P-value=0.48649) respectively between controls and benign tumors, controls and malignant tumors and between benign and malignant tumors.

Neutrality tests

Under the assumption of positive selection (dN>dS) the probability values for controls and benign and malignant tumors are respectively (0.01), (0.037) and (0.064) with p (1.023), (1.802) and (1.529) values that are not significant. Based on

these results, the initial hypothesis is not accepted; therefore the substitutions at the level of exon 15 of the BRAF do not follow the positive selection. For Tajima D, the values for controls (-0.90996; p=0.2), TB (-0.99594; p=0.154) and TM (-0.86541; p=0.211) are not significantly negative. The D* and F* tests of (Fu & Li) and the H of (Fay & Wu) are negatively insignificant for all three groups; the values are reported in Table 5. Unlike the other indices for those of R2 de Ramos, the control values (0.16074; p=0.000), TB (0.16370; p=0.000) and TM (0.16088; p=0.000) are significantly positive.

Mismatch distribution analysis

The disparity of distribution (Mismatch distribution), base pairs for exon 15 of the *BRAF* gene between the three groups, shows the expected and observed frequencies (solid and dotted line respectively) of the differences per pair between the samples (Figure 3). Under the assumption of a constant population and an expanding population, we have a unimodal distribution for controls. However, the distribution is multimodal for benign and malignant tumors.

Diversity indices	Controls	Benign tumors	Malignant tumors
Number of sequences, n	12	31	35
Number of sites, N	181	181	181
Monomorphic sites	173	164	161
Polymorphic sites	8	17	20
Singleton variable sites	6	10	9
Parsimony informative sites	2	7	11
Total number of mutations, Eta	10	22	26
Total number of singleton mutations Eta (s)	8	17	20
Number of haplotypes, h	6	18	23
Average number of nucleotide differences (k)	2.045	3.009	3.615
Transitions (%)	26.51	21.18	46.82
Transversions (%)	73.42	78.82	53.16
R (transition rates/ transversal rates)	0.318	0.238	0.807
Haplotypical diversity (hd)	0.818	0.920	0.934
Nucleotide diversity (π)	0.01130	0.01662	0.01997

Table 2: Values of genetic diversity indices for each population

Amino acids	Controls	Benign tumours	Malignant tumours	P-value T vsTB	P-value T vs TM	P-value TB vs TM
Ala	5.15	5.07	5.15	0.9795	1	0.9795
Cys	0.14	0.05	0.05	0.8363	0.8363	1
Asp	5.01	5.02	5.00	0.9974	0.9974	0.9948
Glu	5.01	5.02	5.00	0.9974	0.9974	0.9948
Phe	9.74	9.49	9.68	0.9522	0.9886	0.9636
Gly	6.68	6.69	6.67	0.9977	0.9977	0.9955
His	3.34	3.34	3.34	1	1	1
Ile	10.15	10.30	10.15	0.9721	1	0.9721
Lys	5.01	4.96	4.81	0.987	0.9478	0.9608
Leu	11.82	12.03	11.96	0.9635	0.9756	0.9878
Met	1.67	1.73	1.76	0.9738	0.9609	0.9871
Asn	0.00	0.05	0.10	0.823	0.7518	0.8972
Pro	1.67	1.94	2.05	0.886	0.8424	0.9556
Gln	3.34	3.34	3.34	1	1	1
Arg	3.34	3.40	3.34	0.9812	1	0.9812
Ser	10.15	10.30	10.30	0.9721	0.9721	1
Thr	6.68	6.47	6.34	0.9522	0.9224	0.9701
Val	5.01	5.02	5.00	0.9974	0.9974	0.9948
Trp	3.34	3.34	3.34	1	1	1
Tyr	2.78	2.43	3.34	0.8765	0.8182	0.7007

Table 3: Frequencies of BRAF amino acids

Groupes	Intra group genetic distances	Inter group genetic distances	Fst
Controls	0.01		0
Benign tumors	0.02	0.0139	P-value=0.86486
Controls			0
Malignant tumors	0.02	0.0159	P-value=0.76577
Benign tumors			0
Malignant tumors		0.0186	P-value=0.48649

Table 4: Intra- and inter-group genetic distances and differentiation index (Fst)

Parameters	Controls	Benign tumors	Malignant tumors	P-value Controls	P-value TB	P-value TM
dN/dS	0.01	0.037	0.064	1.023	1.802	1.529
D de Tajima	-0.90996	-0.99594	-0.86541	0.2	0.154	0.211
D* de Fu et Li	-0.06286	-0.07713	-0.11543	0.46000	1.13637	0.48800
F* de Fu et Li	-0.07359	-0.05006	-0.06326	0.47100	0.45200	0.45500
H de Fay et Wu	-0.14000	-0.06436	-0.22133	0.35400	0.33500	0.33300
R2	0.16074	0.16370	0.16088	0.000	0.000	0.000

Table 5: Values of the Selection Signature Tests

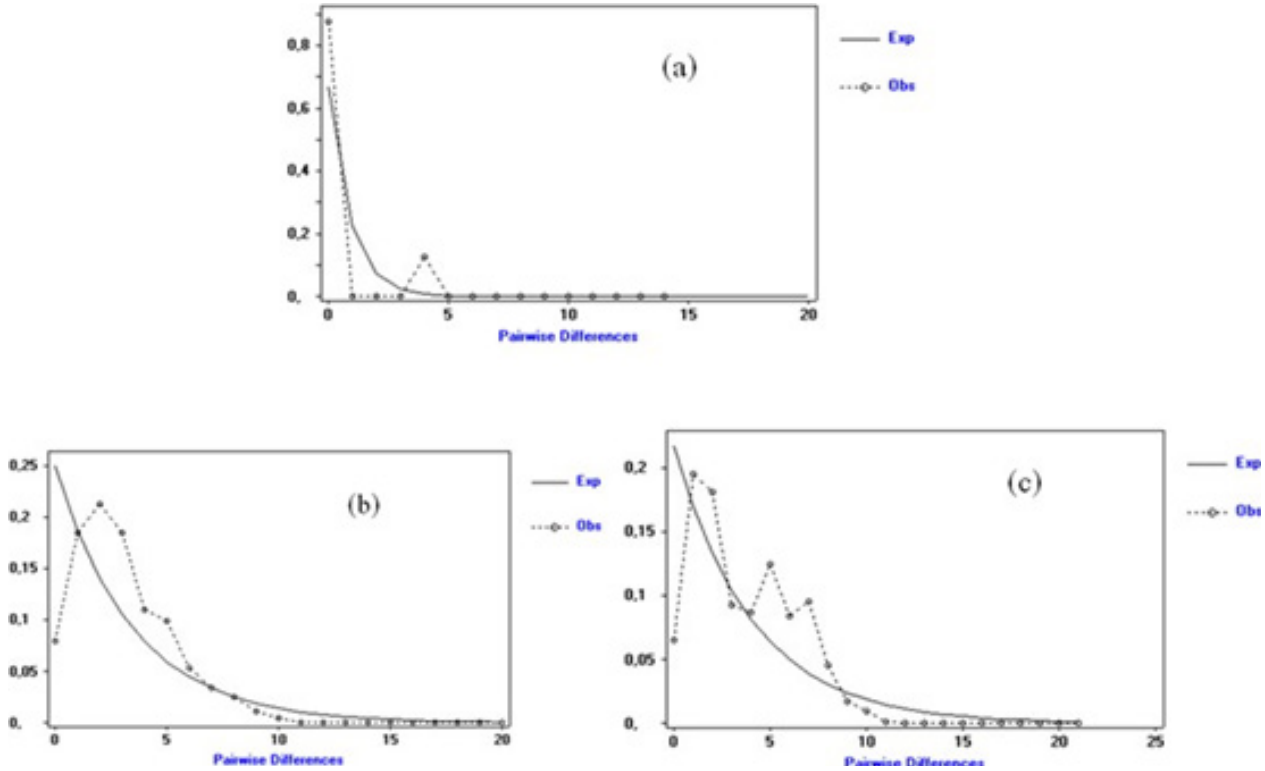


Figure 3: Mismatch curves distribution of controls (a), benign tumors (b) and malignant tumors (c) The SSD, control (0.01093; p=0.55000), TB (0.00346; p=0.59000) and TM (0.00988; p=0.48000) values are positive and not significant for the three groups (Table 6). The values of Rag, controls (0.05005; p = 0.78000), TB (0.02122; p = 0.83000) and TM (0.03150; p=0.36000) are positive and not significant (Table VII).

Parameters	Controls	Benign tumors	Malignant tumors
SSD	0.01093 P-value= 0.55000	0.00346 P-value= 0.59000	0.00988 P-value= 0.48000
Rag	0.05005 P-value= 0.78000	0.02122 P-value= 0.83000	0.03150 P-value=0.36000

Tables 6: Values of SSDs, Rags, and their P-values

Discussion

We have identified mutations, studied genetic variability, genetic differentiation, and genetic evolution in order to determine the involvement of exon 15 of the *BRAF* gene in breast cancer in Senegalese women. 35 patients with breast cancer and 31 patients with benign breast tumors were included in this study. The data from these patients were compared to 12 control subjects used as controls. *BRAF*, which is a nuclear gene, is part of the RAF-MEK-ERK signaling cascade that regulates cell growth, proliferation and differentiation in response to stimulation by growth factors, cytokines and hormones [13]. For this role that plays in the cell justifies the study of the variability of its exon 15 in the context of cancer.

The mutation identification analysis of exon 15 of the *BRAF* gene showed us in benign tumors, the presence of mutations L588H and D598V which correspond to a change of amino acid in position 588 in *BRAF*, with a leucine (L) replaced by a histidine (H) and D598V to a change of amino acid in position 598 in BRAF with an aspartic acid (D) replaced by a valine (V). It has been shown by [14] that *BRAF* activating mutations are common in some benign tumors, such as scalloped colonic polyps, where their frequency reaches 51%, and melanocytic nevi. We observe a synonymous mutation A598A in malignant tumors. These results are different from the substitution of *BRAF* V600E resulting in the substitution of glutamic acid by valine, which accounts for 80% of mutations and is thought to be involved in 66% of malignant melanomas [3], and also involved in several other cancers [15]. In most cases, B-raf mutations are located in exon 15 [16]. In our study of the variability of exon 15 of the *BRAF* gene, several mutations have been described both in malignant and benign tumour sequences. This increased diversity in malignant tumors is reflected in a high number of variable sites (20) compared to TB (17) and controls (8). This justifies that the ability of tumour cells to invade and colonize distant sites is a major characteristic differentiating malignant and benign cancers [17]. We had transition percentages (46.82%) at the malignant tumour levels that are higher compared to benign (21.18%) and control (26.51%) tumors. Our results are in line with those of [18] working on the diagnosis of melanoma. The sequencing of *BRAF* showed the presence of very many mutations and 80% of the mutations were C > Transitions.

A comparative analysis of the genetic distance and degree of genetic differentiation (Fst), intra and inter healthy tissue, benign tissue and cancer tissue, of exon 15 of the *BRAF* gene was performed in a series of 78 patients with breast tumors. The analysis of genetic distances between controls and malignant tumors and benign and malignant tumors revealed a low respective genetic diversity (d=0.0159), (0.0186), but greater than that between controls and benign tumors (d=0.0139). And also we find that the degree of genetic differentiation shows a negative and insignificant Fst (-0.02652; P- value=0.86486), (-0.01890; P-value=0.76577) and (-0.00294; P-value=0.48649) respectively between controls and benign tumors, controls and malignant tumors as well as between benign and malignant tumors. We also found that within malignant and benign tumors, the genetic distance values are equal (d=0.02), higher than those of the controls (d=0.01). This shows that cancer cells have a different property from healthy cells [19], and it may also explain that during carcinogenesis, cancer-related changes the internal structures of the cells but also their environment. Cancer cells have been shown to be less rigid than normal cells due to a reorganization of the cytoskeleton [20]. Breast cancer cells deform more than non-cancerous cells [21].

The analysis of inter-tissue protein diversity by highlighting amino acid variations indicates that, despite slight variations in amino acid frequencies between the three groups, no significant difference could be determined. This can be explained by the aspect of the nuclear DNA gene that has less replication than the mtDNA gene.

Conclusion

Genetic mutations and molecular pathway activation play a vital role in tumour formation. The analysis at the identification of mutation of exon 15 of the BRAF gene led us to conclude that BRAF was mutated

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