Descriptive analysis of KRAS and BRAF mutations in senegalensis patients with colorectal cancer

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ABSTRACT

Worldwide Colorectal cancer (CRC) is a leading cause to cancer mortality and morbidity. In many studies, mutations in the KRAS and BRAF genes are involved in colorectal cancer, and are associated with the primary resistance to the EGFR inhibitors. Objective of study is to investigate the prevalence of KRAS and BRAF mutations in first in Senegal and to correlate clinico-anatomical parameters according to genes mutation status in colorectal cancer. Genomic DNA from the tumor tissue was performed using the ReliaPrep gDNA Tissue kit from Promega on surgical specimens of twenty patients with colorectal cancer. The median age was 55 years and sex ratio was 0.82. Analysis of mutations in codons 12 and 13 of KRAS and in codon 600 of BRAF was assessed using High -resolution melting (HRM). Mutations were detected for KRAS in 45% and for BRAF in 55% of specimens and were not associated with clinico-anatomical parameters. Tumors harboring mutation in both KRAS and BRAF were observed in 15% of cases. The colon location of the tumor was the most recovered. Stage pT3 and pT4 were in more than ¾ of the cases and liver metastases had in two cases.

Keywords: BRAF, colorectal cancer, KRAS, mutation, Senegal.

INTRODUCTION

Colorectal cancer is a common disease in all countries; it remains more of a public health problem in developed countries because of its prevalence and the number of deaths. It’s the third most common cancer in the world after bronchopulmonary and breast cancers (Bray et al., 2011). In developing countries the incidence is low; this is the case in Africa with a rate of 1 to 3% of cancers despite disparities between countries (Bray et al., 2015). In Senegal, the prevalence remains low, but the number of colorectal cancer cases continues to increase (Abdou et al., 2016, Ba et al., 2012).
Indeed, colorectal cancer results from a succession of genetic alterations that affect certain oncogenes, tumor suppressor genes or DNA stability genes. Approximately 70% of colorectal tumors are adenocarcinomas resulting from the transformation of a pre-existing adenomatous polypl (Jass, 2002), (Fearon and Vogelstein, 1990). The mechanisms involved in colon carcinogenesis have been established since 1990 (Fearon and Vogelstein, 1990), and the knowledge of intracellular signaling pathways has made it possible to set up targeted therapies, with the anti-EGFR molecules. However, the efficacy of the latter is compromised by mutations of the KRAS and BRAF genes on the EGFR signaling pathway that are responsible for resistance to these anti-EGFR therapies (Lievre, 2010), (Amrani Hassani Joutei et al., 2013, Amado et al., 2008).

The KRAS gene is a proto-oncogene frequently activated in cancers, and particularly in colorectal cancers, where it is mutated in 35 to 40% of cases (Bardelli A et al., 2002). Point mutations of KRAS affect more than 90% of cases codons 12 and 13 of exon 2 more rarely codons 61 and 146. It is very close to the BRAF gene, also a proto-oncogene, located in the same signaling pathway. The BRAF gene is mutated in 5 to 15% of cases in colorectal cancer and most of the mutations are located at codon 600 of exon 15 (Bos, 1989, Baldus et al., 2010). Concurrent mutations of the KRAS and BRAF genes in the same colorectal tumor have been reported in several studies (Biesmans et al., 2008, Chowdhri et al., 2009). These mutations result from the activation of the EGFR (Epidermal Growth Factor Receptor) receptor, which leads to the expression of growth-promoting genes Berg and (Berg and Soreide, 2012). To our knowledge, molecular research is not yet documented in Senegal.

The objective of our study is to investigate the prevalence of KRAS and BRAF gene mutations and to correlate the mutational status of these genes with clinical-anatomical parameters in patients with colon or rectal cancer in Senegal.

MATERIALS AND METHODS

It is a prospective, descriptive and analytical study, which is carried out over a 12-month period from January to December 2017. All patients are prior informed written consent.

Study Population

We collected tissue specimens from surgical departments and pathology laboratories of the General Grand Yoff and Aristide Le Dantec hospitals where patients underwent colorectal cancer resections. Tumor tissue stored at -80°C before use at the Biochemistry Department of Aristide Le Dantec Hospital. Molecular analysis was performed in molecular biology unit of the same hospital.

DNA Extraction

DNA genomic (gDNA) isolation was performed using the Promega ReliaPrep gDNA Tissue kit protocol. Approximately 25 mg of tissue were collected in Eppendorf vials with 160 µl of PBS. The sample was resuspended with 20 µl of proteinase K and 200 µl of tissue Lysis Buffer. The sample was gently mixed for 10 seconds, incubated at 56°C for 1 hour under agitation. At the end of the incubation, 20 µl of RNase A are added and then mixed for 10 seconds and incubated again at 56°C. for 10 minutes. Then 250 µl of Binding Buffer were added and all was mixed for 10 seconds and centrifuged at 14000 g for 1 minute. DNA was extracted with ReliaPrep column in a collection vial. DNA was eluted with 100 µl of PCR water after centrifugation and washing series. Purity and concentration of gDNA were determined with NanoDrop One (Thermo Fisher Scientific). DNA was stored at -20°C before use.

High-Resolution Melting Analysis

HRM analysis was performed using the light Cycler 480 II HRM master kit (Roche Diagnostics®). It allows to detect mutations of exon 2 KRAS including codons 12 and 13 and codon 600 of BRAF. Oligonucleotides primers used were KRAS (92pdb) sens: TATAAGGCCTGCTGAAATGACTGA and antisens: GAATTAGCTGTATCGTCAAGGCACT and for BRAF (147pdb). sens: GGTTGTGGTCTAGCTACAG and antisens: AGTAATCGAGCATCTCGAG).

All DNA samples were reduced to 15 ng/ml by dilution with pure water. Forty nanograms (30 µg) of gDNA was amplified in a final volume of 20µl by using the following: 2.4 µl of MgO2 (3 mM), 0.4 µl of sense and antisense primer (10 nM), 10 µl of Master Mix (2X) (containing the Taq polymerase, the dNTPs and the fluorescent intercalating agent), 4.8 µl of water. The mixture is pipetted and dispensed along the plate. The quality of handling was appreciated with water, a positive control and a negative control. The plate is then covered with transparent film and then centrifuged for 2 min at 500 g and deposited on the light Cycler 480 II Roche®.

The HRM assay protocol requires initial denaturation at 95°C for 5 min followed by 50 PCR cycles of 15 sec at 95°C, 15 sec at 68°C and 20 sec at 72°C. For the melting curve, samples are denatured with an initial hold of 1 min at 95°C and 1 min at 40°C and a melting profile from 65°C to 95°C with a ramping degree of 0.02°C/sec. The analysis was performed in duplicate. HRM analysis allows determining the mutated and non-mutated profile (Figures 1A and 1B).
Figure 1: HRM melting curves fusion of the research KRAS and BRAF genes mutations.

The fusion temperature of mutated samples is weaker than the fusion temperature of non-mutated samples.

Statistical Analysis

Statistical analyses were performed by using Microsoft Excel software 2013. Fisher’s test was used to compare interaction between genes mutation status and data (sex, age, tumor location, disease stage). P<0.05 was considered statistically significant.

RESULTS

We first characterized our population and also presented analytical data.

Characteristics of study population

The study population consisted of 20 patients, 45% men and 55% women with a sex ratio of 0.82. The average age of the study population was 55 years with extremes of 33 years and 73 years. For two patients, age was not defined. The majority of patients were in the 61-70 age group (33%). These results are summarized in Figure 2.

Analytical data: Results will be presented according to tumor location, TNM classification, mutation frequencies and correlation between data.

Tumor location: Tumor location was predominated in left colon. Two patients had liver metastases. For five patients, histopathological data were not found (Figure 3).

Figure 3: Tumor location.

TNM classification: The colorectal cancers found were all differentiated adenocarcinomas. According to the international TNM classification of these adenocarcinomas, the pT3 stage was largely predominated (72%) (Figure 4).

Figure 4: TNM classification of adenocarcinomas.

Analysis of the HRM fusion curves showed that KRAS mutations were detected in 45% and BRAF mutations in 55% of the specimens. A coexistence of 15% of mutations of both two genes was found (Table 1).

Table 1: KRAS and BRAF mutations prevalence.

<table>
<thead>
<tr>
<th>Mutation Status</th>
<th>Number</th>
<th>Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>KRAS mutant</td>
<td>9</td>
<td>[45%]</td>
</tr>
<tr>
<td>BRAF mutant</td>
<td>11</td>
<td>[55%]</td>
</tr>
<tr>
<td>Concomitant KRAS and BRAF mutations</td>
<td>3</td>
<td>[15%]</td>
</tr>
<tr>
<td>Wild -type</td>
<td>3</td>
<td>[15%]</td>
</tr>
</tbody>
</table>

Clinico-Anatomical Characteristics According to KRAS and BRAF Mutations Status

Table 2 shows the relationship between the mutational status of the KRAS and BRAF genes with characteristics (gender, age) and with clinico-
anatomical features (tumor location, metastasis and tumor stage according to the TNM classification) (Table 3).

**Table 2**: Correlation mutational status of KRAS and BRAF genes with clinico-anatomical features.

<table>
<thead>
<tr>
<th>Clinico-anatomical features</th>
<th>Total No</th>
<th>KRAS P-value</th>
<th>KRAS P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Wild-type Mutant</td>
<td>Wild-type Mutant</td>
<td></td>
</tr>
<tr>
<td>Total No of patients</td>
<td>20</td>
<td>11 9</td>
<td>9 11</td>
</tr>
<tr>
<td>Gender</td>
<td>1</td>
<td>0.65</td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>9</td>
<td>5 4</td>
<td>5 4</td>
</tr>
<tr>
<td>Female</td>
<td>11</td>
<td>6 5</td>
<td>4 7</td>
</tr>
<tr>
<td>Years</td>
<td>30-40 Years</td>
<td>4 2 2</td>
<td>3 1</td>
</tr>
<tr>
<td></td>
<td>41-50 Years</td>
<td>2 0 2</td>
<td>1 1</td>
</tr>
<tr>
<td></td>
<td>51-60 Years</td>
<td>5 3 2</td>
<td>1 4</td>
</tr>
<tr>
<td></td>
<td>61-70 Years</td>
<td>6 4 2</td>
<td>4 2</td>
</tr>
<tr>
<td></td>
<td>71-80 Years</td>
<td>1 0 1</td>
<td>0 1</td>
</tr>
</tbody>
</table>

**Table 3**: Correlation mutational status of KRAS and BRAF genes with clinico-anatomical features.

<table>
<thead>
<tr>
<th>Clinico-anatomical features</th>
<th>Total No</th>
<th>KRAS P-value</th>
<th>KRAS P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Wild-type Mutant</td>
<td>Wild-type Mutant</td>
<td></td>
</tr>
<tr>
<td>Total No of patients</td>
<td>20</td>
<td>11 9</td>
<td>9 11</td>
</tr>
<tr>
<td>Tumor location</td>
<td>1</td>
<td>0.47</td>
<td></td>
</tr>
<tr>
<td>Right colon</td>
<td>3</td>
<td>1 2</td>
<td>1 2</td>
</tr>
<tr>
<td>Left colon</td>
<td>9</td>
<td>4 5</td>
<td>5 4</td>
</tr>
<tr>
<td>Rectum</td>
<td>3</td>
<td>2 1</td>
<td>0 3</td>
</tr>
<tr>
<td>Metastasis</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>No</td>
<td>13</td>
<td>7 6</td>
<td>4 9</td>
</tr>
<tr>
<td>Yes</td>
<td>2</td>
<td>1 1</td>
<td>1 1</td>
</tr>
<tr>
<td>TNM stage</td>
<td>0.74</td>
<td></td>
<td>0.74</td>
</tr>
<tr>
<td>pT2</td>
<td>3</td>
<td>2 1</td>
<td>1 2</td>
</tr>
<tr>
<td>pT3</td>
<td>10</td>
<td>4 6</td>
<td>4 6</td>
</tr>
<tr>
<td>pT4</td>
<td>1</td>
<td>0 1</td>
<td>1 0</td>
</tr>
</tbody>
</table>

The values of p obtained are all greater than 0.05. A comparison between mutational status of genes and features did not reveal any significant difference.

**DISCUSSION**

We conducted this first study in Senegal to assess the frequencies of mutated genes KRAS and BRAF in colorectal cancers. HRM is a technique for rapid screening of population by determining the status of "mutated" or "non-mutated" point mutation. HRM allows the characterization of DNA samples by their double-to-single-strand dissociation behavior following a rise in temperature. Analysis of the sample fusion curves showed 45% prevalence of KRAS gene mutation. Our results are similar to those of many published studies. In fact, frequencies of 30 to 50% of the KRAS gene at codons 12 and 13 of exon 2 have been identified (Diallo-Agne, 2013, Athanasiadis et al., 2015, Ajdarkosh et al., 2016).

Nevertheless, lower KRAS gene mutation rates below 30% are described in studies in Africa. Notably in Morocco, Tunisia and Nigeria with respectively KRAS gene mutation rates of 15.38%, 21% and 23.91% (Amrani Hassani Joutei et al., 2013, Abdelmaksoud-Dammak et al., 2015, Abdulkareem et al., 2012). Whereas for the BRAF gene, the prevalence of mutations was 55%. It is far from the results found in the literature (Athanasiadis et al., 2015); as evidenced by the reported prevalence of studies in Nigeria and Morocco at 4.5% and 5.43%, respectively (Abdulkareem et al., 2012) (Amrani Hassani Joutei et al., 2013).

However, a concomitant mutation of the two KRAS and BRAF genes of 15% was found. Indeed, most studies show that mutations of these genes are mutually exclusive (Bardelli et al., 2002), (Athanasiadis et al., 2015). Others seem to confirm the opposite with the results of the works of (Aghdaei et al., 2017) and (Amrani Hassan Joutei et al., 2013), each with a case of coexistence. However, we did not obtain a correlation between the mutational status of the KRAS and BRAF genes, nor with gender nor with age. This has been reported by other works (Chowdhri et al., 2009), (Baron et al., 2013, Gorukmez et al., 2016).

Our results showed a predominance of women with a sex ratio of 0.82. The average age was 55 but the majority of the study population was older than 60 (Figure 2). A female predominance has been found in other studies with a high average age (Baron et al., 2013, Selcukbircik and Serdengecti, 2013). These results confirm the data obtained in a previous study of 41 patients in our department, the majority of whom were over 60 years old, with an average age of around 50 years and a sex ratio of 0.95 (Abdou et al., 2016). The same observations were found in a study in Togo for an average age of the same age, which average exceeds 60 with (Chowdhri et al., 2009) and (Amegbor et al., 2014). On the other hand, in the African literature, works have shown a predominance of men,
especially those carried out in Togo and Tunisia (Chowdhri et al., 2009, Amegbor et al., 2014). This male pre-dilection of colorectal cancer has been found in other non-African studies, but with a higher average age around 70 years (Diallo Agne, 2013, Benson 2007). Despite contradictory results on gender and age, it is generally accepted that aging, or more precisely advanced age, is a risk factor for the occurrence of colorectal cancer.

Moreover, our results did not show a relationship between the presence of mutation and the location of the tumor. This observation was also reported by (Hunt et al., 2011) and (Amirfallah et al., 2014). However, work of (Athanasiadis et al., 2015) and (Buchanan et al., 2013), showed significant correlation between left tumor location and mutational status, and more KRAS and BRAF mutations on the right colon. Like many studies in the literature Ndiaye et al. (2016), Marchoudi et al. (2013) and Samara et al. (2015), our results show a left colon location of the tumor; while for others, colorectal cancer has more rectal than colon location Selcukbircik et al. (2013), Nawai et al. (2009).

In our study, the colorectal cancers found were all well differentiated adenocarcinomas and the tumor stage was reported according to the international TNM classification. We found the pT2, pT3 and pT4 stages with respectively 21%, 72% and 7%. The pT4 stage, the most advanced stage, was weakly observed in our patients. In contrast, the pT3 and pT4 stages accounted for almost 80% of the study population. Our results are similar with many studies (Abdou et al., 2016, Ajdarkosh et al., 2016, Amegbor et al., 2014).

The advanced stage of adenocarcinoma tumors of our patients could result from delayed diagnosis of colorectal cancer. The latter makes it possible to justify the occurrence of metastases (13%) all liver in our study.

This can be explained by the longer or shorter delays observed by patients before having recourse to a medical consultation. In Africa, this phenomenon is often observed because many people first consult practitioners of traditional medicine before presenting themselves in hospital structures (Diallo Owono et al., 2011).

CONCLUSION
The prevalence of BRAF and KRAS gene mutations is high in our study population. However, it is necessary to expand the sampling and confirm the result by sequencing.

REFERENCES


