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First Detection of KRAS Mutation in Colorectal Cancer Patients in Côte d'Ivoire

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ABSTRACT

Advances in molecular biology tools have made it possible to make progress in terms of therapeutics by acting specifically on the molecular mechanisms involved in the virulence of pathogens or in the development of the disease. In the case of cancer, new therapeutics have been developed thanks to these advances. This is called targeted therapy. Targeted therapy molecules specifically act as a molecule, or a molecular pathway involved in the development of the tumor. The use of targeted therapy drugs therefore requires molecular characterization of tumors. In developing countries, the reference tool is high-throughput sequencing, but due to the high cost, this strategy remains inaccessible for the majority of African populations. It remains necessary to implement alternative molecular diagnostic tools in order to reduce the disparities currently observed in access to cancer care. The High-Resolution Melting analysis (HRM) method is based on real-time PCR and is described as a simple, rapid and specific method for the detection of somatic mutations predictive of anti-tumor therapeutic response. We demonstrated in this study that this method could be implemented in a lower middle-income country like Côte d'Ivoire, and we used it to detect KRAS mutations in colorectal cancer patients for the first time in Côte d'Ivoire.

Keywords: cancer, Côte d'Ivoire, KRAS gene mutations, molecular diagnosis, PCR-HRM.

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I. INTRODUCTION

Cancer is a global public health problem, and this is especially true for sub-Saharan African countries. According to the WHO, the burden of cancer on African populations is expected to double by 2040 due to population growth and aging [1]. The Lancet oncology commission, sub-Saharan Africa consider that still has many gaps to fill in addition to

access to treatment. These include gaps in data collection and analysis, early diagnosis and patient screening [2]. Screening patient is important for an adequate and effective early treatment in a context where most of cancers are diagnosed at a metastatic stage [3]. Colorectal cancer (CRC) is the third cancer worldwide affecting both sex according to GLOBOCAN 2020 [2]. CRC is characterized by several genetic mutations resulting in anarchic multiplication of cells

in the inner lining of the colon and rectum [3]. These mutations can be induced by many factors among which are environmental and genetic ones. Mutations involved in the development of CRC can be located at different genes. However, the most frequently gene mutation concern the KRAS (Kirsten Rat Sarcoma virus) gene which is involved in 50% of CRC cases [4].

The proto-oncogene KRAS is a signal transducer of cell survival and proliferation pathways and is frequently activated in cancers [5]. This activation of the protein is related to the introduction of mutations into the gene and it has been shown that the presence of mutations in the KRAS gene leads to cell resistance to certain anti-tumor treatments [6], [7]. Studies showed that the KRAS gene is mutated in 35 to 40% of colorectal cancer cases [8]. Finally, the analysis of the KRAS gene in colorectal cancer and lung cancer demonstrated that mutations occur mainly in codons 12 and 13 of exon 2 of the gene [9]. If the detection of these mutations has no real prognostic value but has a strong predictive value of response or nonresponse to targeted therapy treatment. This justifies that European and North American countries recommend the KRAS test before therapy is established for patients with metastatic colorectal cancer [10], [11].

Targeted therapy is an approach used in addition to chemotherapy, radiotherapy and surgery. This involves the use of molecules that specifically target a receptor or metabolic pathway directly involved in the development of the disease. Targeted therapy treatments are available in Côte d'Ivoire but biological tests for the detection of mutations in the KRAS gene are not implemented yet.

In industrialized countries, the strategy used to search for somatic mutations with prognostic or predictive value in the case of cancer is high-throughput sequencing. In sub-Saharan Africa, however, this strategy would be too expensive for our populations.

High resolution melting curve analysis (HRM) is a method based on the real-time PCR technique which has the advantage of being simple, fast and less expensive than sequencing [11]–[13]. The HRM method allows the detection of the presence or absence of a mutation in a target DNA region. During the real-time PCR reaction, the amplification of the target region is visualized thanks to the presence of fluorophore in the reaction mix. At the end of the amplification cycle, the generated DNA double strands are subjected to a progressive increase in temperature leading to the separation of the double strands and the decrease of the fluorescent signal. The introduction of a mutation in the reference sequence leads to a modification of the melting temperature of the DNA which can be detected by using a specific fluorophore. The use of the HRM method allows a first screening between samples with and without a mutation. However, the nature of the mutation must be determined by the Sanger sequencing method. This two-step strategy is therefore more advantageous for our population than high-throughput sequencing of all samples.

This study showed for the first time, performed mutation detection in the KRAS gene in Côte d'Ivoire from samples of patients diagnosed with CRC using HRM.

II. MATERIAL AND METHODS

A. Sampling and Samples Pre-Treatment

Samples of patients diagnosed with colorectal cancer after pathological analysis were collected from the Central Laboratory, Plateau, Abidjan. These samples were fixed in formalin and embedded in kerosene between 2019 and 2020. Genomic DNA extracted from HEK 293T cell line were used as wild-type KRAS gene control, whereas genomic DNA extracted from HCT 116 cell line were used as control for mutation in codon 12 of exon 2 of the KRAS gene.

B. DNA Extraction from Formalin Fixed Paraffin – Embedded (FFPE) Tissue

Thirty (30) milligrams (mg) of tissues were taken from each sample. Those tissues pieces were deparaffinized with toluene as described in the DNeasy Blood and Tissue Extraction Kit protocol (Qiagen). Each tissues section was put in a 2 mL microtube and immersed with 1.2 mL of toluene (ROTISOLV®≥99.8%, GC Ultra Grade). After centrifugation at maximum speed for 5 minutes at room temperature, the toluene was removed from the microtube. Tissues Pieces were then washed with 1.2 mL of absolute ethanol, mixed briefly by vortex, and then centrifuged for 5 minutes at 13000 rpm. Ethanol was removed from the microtube, and the washing operation was repeated once more. The microtubes containing the samples were then placed to dry at 37 °C for 15 minutes to evaporate ethanol traces. Genomic DNA extraction was performed using the DNeasy Blood and Tissue Kit (Qiagen) following the manufacturer's instructions.

C. PCR-HRM

The codons 12 and 13 (exon 2) of the KRAS gene were amplified using the following described primers KRAS 2-Fwd: 5'-TCA TTA TTT TTA TTA TAA GGC CTG CTG AA-3' and KRAS 2-Rev: 5'-CAA AGA CTG GTC CTG CAC CAG TA-[12]. PCR amplifications were performed in a StepOnePlus qPCR machine (Applied Biosystems, Foster City, CA) calibrated for the detection of SYTO 9 intercalating fluorescence dye. The reaction mixture consisted of 10 µL of MeltDoctor HRM master mix 2X (ThermoFischer Scientific), 1 µL of each 10 µM primer and molecular biology grade water for final volume of 20 µL. The amplification program was done as follows: one cycle at 95 °C for 15 minutes for enzyme activation, 40 cycles at 95 °C for 15 seconds and then 60.7 °C for 1 minute for sequence amplification; the melting cycle was as follows: 95 °C for 10 seconds, 60.7 °C for 1 minute, 95 °C for 15 seconds and 60.7 °C for 15 seconds. For the melting curve, the temperature increasing gradient slope is 0.3% between 60.7 °C and 95 °C. Results obtained after amplification were analyzed with the High-Resolution Melting (HRM) analysis software (ThermoFischer Scientific). The HEK 293T sample which is known not contain any mutation in the target region is selected as reference and each point of its melting curve is considered as the baseline point for a given temperature. For each temperature, the software determines the fluorescence difference between the reference sample and the other samples, thus generating a differential melting curve. Each sample was performed in triplicate.

D. Sequencing

The PCR-HRM products were used as template for a nested PCR with the following primers KRAS 1-Fwd: 5'-TTA TAA GGC CTG CTG AAA ATG ACT GAA-' and KRAS 1-Rev: 5'-TGA ATT AGC TGT ATC GTC AAG GCA CT-3' described previously [12]. The reaction mixture contained 10 μ L of PCR Master Mix 2X (Promega), 1 μ L of each primer at 10 μ M, and molecular biology grade water for a final volume of 20 μ L per reaction. Amplification was performed in the Vapoprotect Mastercycler Pro thermalcycler (Eppendorf, USA Scientific, Inc.) under the following conditions: one cycle at 95 $^{\circ}$ C for 2 min for enzyme activation, 30 cycles at 95 $^{\circ}$ C for 30 seconds, 67.5 $^{\circ}$ C for 30 seconds, 72 $^{\circ}$ C for 1 min. The products obtained were purified with the ChargeSwitch[®]-Pro PCR Cleanup kit (Invitrogen; Thermo Fisher Scientific, Inc.) before being re-amplified for sequencing. The KRAS 1 primer pair was used to perform a BigDye PCR in a final volume of 20 μ L, the reaction mix was composed of: 2 μ L BigDye buffer 5X (Applied Biosystems, Foster City, CA), 4 μ L BigDye[®] Terminator v3.1 Cycle Sequencing (Applied Biosystems, Foster City, CA), 1 μ L 4 μ M primer, 5 μ L purified DNA and molecular biology grade water. The amplification program was as follows: 25 cycles at 96 $^{\circ}$ C for 30 seconds, 50 $^{\circ}$ C for 15 seconds, 60 $^{\circ}$ C for 4 minutes and then one cycle at 4 $^{\circ}$ C for infinity. The amplification products were purified using the Agencourt CleanSeq Dye-Terminator Remove kit, according to the manufacturer's instructions before detection on a 24 capillary ABI 3500XL Genetic Analyzers (Applied Biosystems, Foster City, CA).

III. RESULTS

The KRAS gene could be amplified in the reference controls corresponding to HEK 293T and HCT 116 lines. This shows that the primers used have good specificity for the targeted region. This was characterized by the unique melting peak observed at the end of the amplification reaction (Fig. 1B). The KRAS gene was well amplified in sample E4 but the gene amplifications in samples E2 and E3 were low. Sample E1 could not be amplified despite several attempts (Fig. 1A).

HRM indicates that the melting curves of all samples differ from that of the reference HEK 293T sample. However, only sample E4 is found to have a curve comparable to that of the HCT 116 control (Fig. 2).

Sample E1 was not sequenced because it was not amplified during HRC-MRP. Even if it had been amplified later, sample E3 could not have been sequenced. All samples were sequenced by the Sanger method to confirm the PCR-HRM results. The PCR-HRM results for the remaining samples (E2, E3, and E4) are shown in Fig. 3.

The result of Sanger sequencing showed that the HEK 293T line contained no mutations in the KRAS gene, whereas the HCT 116 line had a mutation in codon 13 of exon 2 of the gene. This was a substitution of guanine for adenine. Sample E4 had a mutation in codon 12 of exon 2 of the KRAS gene, a substitution of guanine for adenine. No mutations were observed in codons 12 and 13 of exon 2 of the KRAS gene for sample E2.

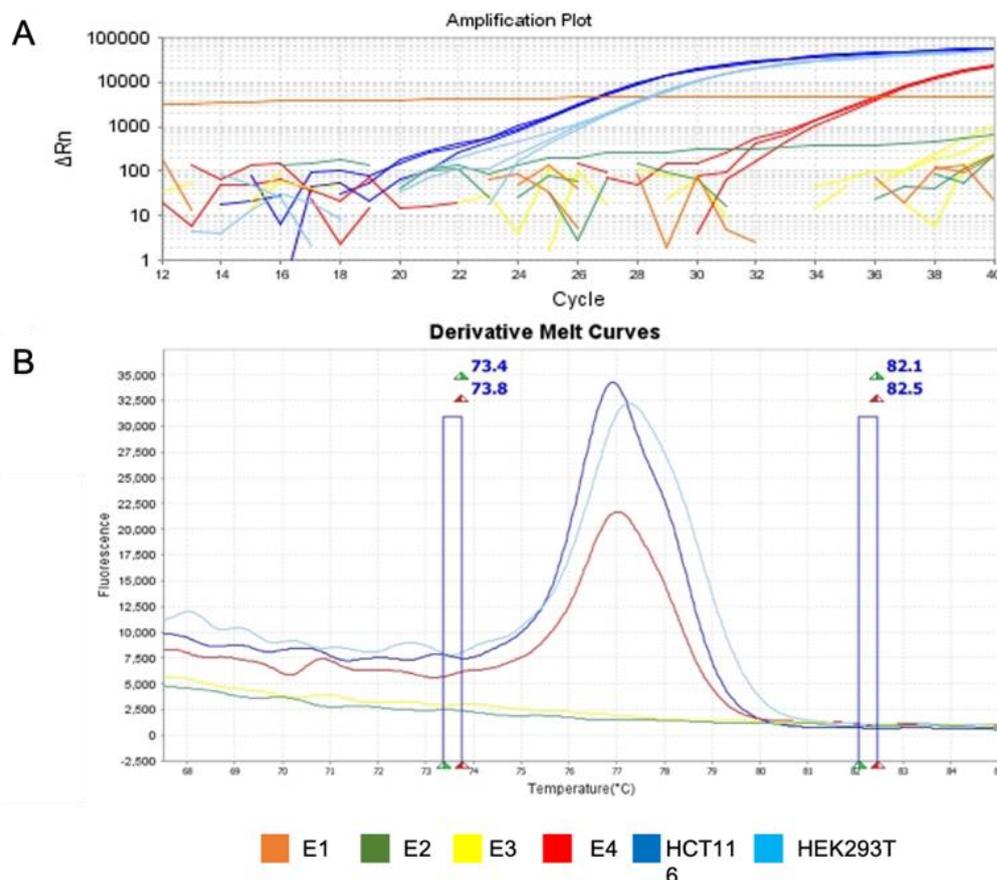


Fig. 1. Amplification result of the samples. A) Amplification curve of the tested samples. B) Melting curve of the amplicons reflecting the specificity of the primers used.

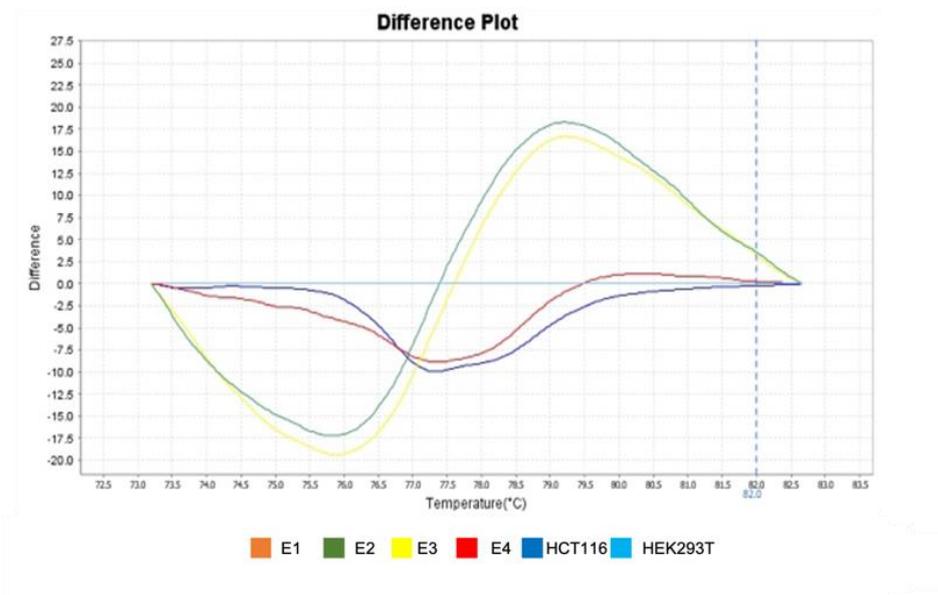


Fig. 2. HRM analysis results. For each temperature the difference between the melting curve of the reference sample and the melting curves of the other samples are shown.

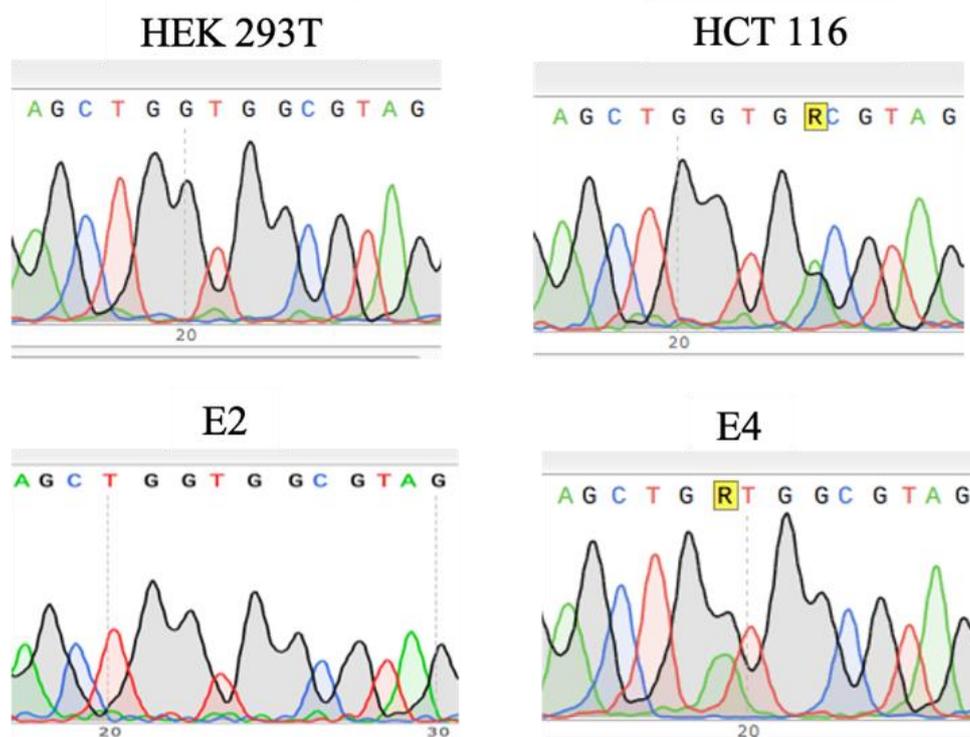


Fig. 3. Sequencing chromatograms.

IV. DISCUSSION

The use of the HRM method allowed to detect a mutation in the KRAS gene in a CRC patient for the first time in Côte d'Ivoire. It was found that the tumor DNA of this patient contained the G12D mutation, the most frequently described mutation in the KRAS gene in patients with metastatic colorectal cancer.

Regarding the E1 sample, it could not be amplified probably due to the quality of the DNA extracted which was not evaluated before amplifications. In a diagnostic context in France, if the analysis is inconclusive, a report is written, and a new sample requested. The samples E2 and E3 were

amplified late with Ct values above 35. Under these conditions the PCR HRM is not validated, and this was confirmed with the melting curve results derived from these amplifications (Fig. 1B) which showed no fusion peak. On the other hand, when the DNA was amplified with a Ct lower than 35, the HRM method allows to highlight the presence or not of a mutation in the target region.

The implementation of the HRM method in Côte d'Ivoire constitutes an alternative to the strategy used in developed countries, which consists of systematic sequencing all samples. In our context, this strategy of direct sequencing presents a major limitation in terms of financial costs for the laboratory and the population due to the cost of purchasing

equipment and the cost of the analysis.

The implementation of the HRM method in Côte d'Ivoire is an alternative to the strategy used in industrialized countries, which consists of systematic sequencing all samples. In our context, this strategy of direct sequencing presents a major limitation in terms of financial costs for the laboratory and the population due to the cost of purchasing equipment and the cost of the analysis.

One advantage of the HRM method is to select samples and only samples of interest containing mutation are sequenced by Sanger and the nature of them determined.

According to forecasts, the burden of cancer on sub-Saharan African countries will double by 2030, so it is imperative for African researchers to initiate projects that will improve the management of our populations from early detection to therapeutic follow-up.

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CONFLICT OF INTEREST

The authors declare no conflict of interest or financial ties to conclude.

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