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Model of Colorectal Cancer for Implementation of the HRMA Method for the Genetic Characterization of Human Pathologies

Safiatou T.G. Coulibaly[1,*], Valérie Mbengue Gbonon[1], Flore B. Diplo[1], David Ngolo Coulibaly[2], Solange Kakou Ngazoa[2], Aboubacar Sylla[2], Mireille Dosso[1,2]

[1]Genetics and Cancer Laboratory, Molecular Genetics Platform, Institut Pasteur of Cote d'Ivoire, 01 BP 490 Abidjan 01, Cote d'Ivoire
[2]Molecular Biology Platform, Institut Pasteur of Cote d'Ivoire, 01 BP 490 Abidjan 01, Cote d'Ivoire
*Corresponding author: safiatoucoulibaly@pasteur.ci

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Abstract *Context:* In developing countries and particularly in Sub-Saharan Africa, access to sequencing techniques is limited. In this context, it is necessary to adopt strategies that will allow researchers to work on molecular genetics and genomics and to allow the greatest number of people to benefit from a precision diagnosis that until now has been outsourced to laboratories in Western countries. The high-resolution curve analysis method (HRMA) for the detection of point mutations in diagnostic choices was evaluated here. *Methodology and Results:* Using genomic DNA from cell lines, the mutation detection sensitivity of the HRMA method was tested on samples containing different percentages of mutated DNA. The results obtained show that the HRMA method can discriminate wild-type samples from those containing a mutation, even for small amounts of mutated DNA in the sample. *Conclusion:* For the time being, systematic sequencing of all samples for research and diagnosis is a very expensive strategy in our context. The present evaluation allows to consider molecular genetic and genomic studies as well as molecular diagnosis in two steps: (i) screening of samples by the HRMA method; (ii) sequencing of samples containing a mutation by the Sanger method.

Keywords: *High-resolution melting curve analysis (HRMA), human pathology, molecular genetics, sequencing, Cote d'Ivoire*

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1. Introduction

Research in Africa has been focused on the early detection of microbial infections and the study of antimicrobial resistance because of the burden of these diseases on the population. With the emergence of molecular technologies, many countries have invested in molecular biology or molecular genetics platforms. These platforms are equipped with instruments that allow rapid and specific detection of pathogens as well as the detection of the presence or absence of resistance genes to guide the therapeutic choice. Molecular platforms are routinely used for the detection and monitoring of antimicrobial resistance genes as well as the monitoring of the viral load of people living with HIV or HBV.

While the mortality rate from non-communicable diseases, and in particular cancer, is still lower than that of communicable diseases such as malaria, yellow fever, or HIV/AIDS, cancer is nevertheless increasing and is now considered as a public health problem in sub-Saharan Africa [1,2,3]. The advantage of countries that have invested in molecular platforms for infectious diseases is that those molecular biology techniques can also be used for better management of patients with non-communicable diseases such as cancer.

Today, in addition to surgery, radiotherapy and chemotherapy, there is targeted therapy to fight tumors. This therapeutic strategy is based on the use of molecules that specifically target a receptor or a metabolic pathway directly involved in the development of the disease. Research on the molecular mechanisms involved in the survival and proliferation of tumors has revealed various metabolic pathways whose activation or inhibition leads to uncontrolled cell growth [4,5,6].

There is no standard method for tumor profiling but the most widely used method in industrialized countries is NGS sequencing. In sub-Saharan Africa, there are few laboratories with such tools and it is a long and time-consuming method. For cases where the desired mutations are localized at specific locations within a gene, it may be advantageous to use an alternative method such as the high-resolution melting curve analysis method HRMA

[7,8,9]. This technique is similar to real-time PCR and allows us to know if the same DNA region from different samples has identical sequences. To do this, after amplification, the PCR products are subjected to a gradual increase in temperature, leading to the denaturation of the generated DNA strands. If the sequences are identical, the melting temperatures of the different segments will be identical, whereas, in the presence of a modification of the nucleic sequence (substitution, insertion, or deletion), the melting temperature will be modified in comparison with the reference sequence. This method is described as fast, sensitive and specific for the detection of point mutations such as KRAS mutations [10,11].

The present work aims to implement the HRM method for the detection of point mutations in Côte d'Ivoire. To do so, we used a model mutation in codons 12 and 13 of exon 2 of the KRAS gene. Mutations in this gene have a strong predictive value in the therapeutic response in the management of colorectal cancer patients and the determination of the genetic profile of this gene is mandatory in industrialized countries before the start of treatment.

2. Material and Methods

2.1. Genetics Material

Genomic DNA extracted from two cell lines from which the genetic profile of the KRAS gene is known was used.

The HEK 293T cell line is a human embryonic kidney cell line. This cell line contains a wild-type KRAS gene, whereas the HCT 116 cells are derived from human colon cancer and have a mutation in codon 12 of exon 2 of the KRAS gene. Amplification and melting curve analysis of different types of samples were performed to determine the sensitivity of the HRMA method. The different samples tested were done according to Table 1.

2.2. HRMA

The sequence of primers used for target region amplification is shown in Figure 1, which had been described previously [8]. Amplification of the target region was performed with a StepOnePlus™ real-time PCR machine (Applied Biosystems™). The reaction mix contained 10µL MeltDoctor™ HRM Master Mix, 1 µL of sense primer (10 µM), 1 µL of antisense primer (10 µM), 20ng of genomic DNA, and sufficient ultrapure water for 20 µL. Amplification and amplicon melting parameters were: 1 cycle at 95°C for 15 minutes then 40 cycles of 95°C for 15 seconds and 60.7°C for 1 minute for the KRAS 2 primer pair then 95°C for 10 seconds, 60.7°C for 1 minute, 95°C for 15 seconds and 60.7°C for 15 seconds. During this melting phase, the temperature increased from 60.7°C to 95°C at a rate of 0.2°C per second. All samples were tested in triplicate.

2.3. HRMA Results Analysis

The HRMA results were analyzed using High-Resolution Melt Software v3.1 (Applied Biosystems™). Amplification and melting curves were used to control amplification. The high-resolution melt curves along with the differential curve generated by the software were used to analyze the sensitivity of the method. The melting curve of the "non-mutated DNA" sample (100% HEK293T) is considered as a reference. The software determines for each temperature point the difference in fluorescence between the other samples and the "non-mutated DNA" sample. Each sample was analyzed in duplicate, for the graphical representations the average of the two results was calculated for each sample.

Table 1. composition of the different samples tested

	ADN non muté	3% ADN muté	5% ADN muté	10% ADN muté	20% ADN muté	50% ADN muté	ADN muté
HEK 293T	100%	97%	95%	90%	80%	50%	
HCT 116		3%	5%	10%	20%	50%	100%

Figure 1. Representation of a portion of the KRAS gene. Boxed in red, codons 12 and 13 of exon two of the gene. Purple box: the sequence of primers used for the amplification

3. Results

The amplification curve (Figure 2A) shows that the primers used for amplification of the area of interest work well. All samples except the negative control reaction that contained ultrapure water instead of genomic DNA show amplification signals. The presence of a single melting peak in Figure 2B reflects the specificity of the primers used. These primers do not hybridize to any other region of the genome, in which case multiple melting peaks would have appeared.

On the high-resolution melting curve (Figure 3A) differences are observable for the different samples, however, a differential analysis by the software is necessary to have a clearer view and to know if the differences are significant or not. After analysis by the software (Figure 3B) it can be seen that the more mutated DNA the sample contains, the greater the difference between its melting curve and that of the reference sample, in this case the HEK 293T sample (light blue). In many practical cases, the study target may not be a pure sample. In the case of cancer analyses, cancer cells are most often in the minority in the sample compared to normal cells. It was therefore desirable to determine whether the method was sensitive enough to detect mutated DNA in a mixed sample. Figure B shows that under our experimental conditions, the method can discriminate a sample containing no mutated DNA from a sample containing only 3% mutated DNA.

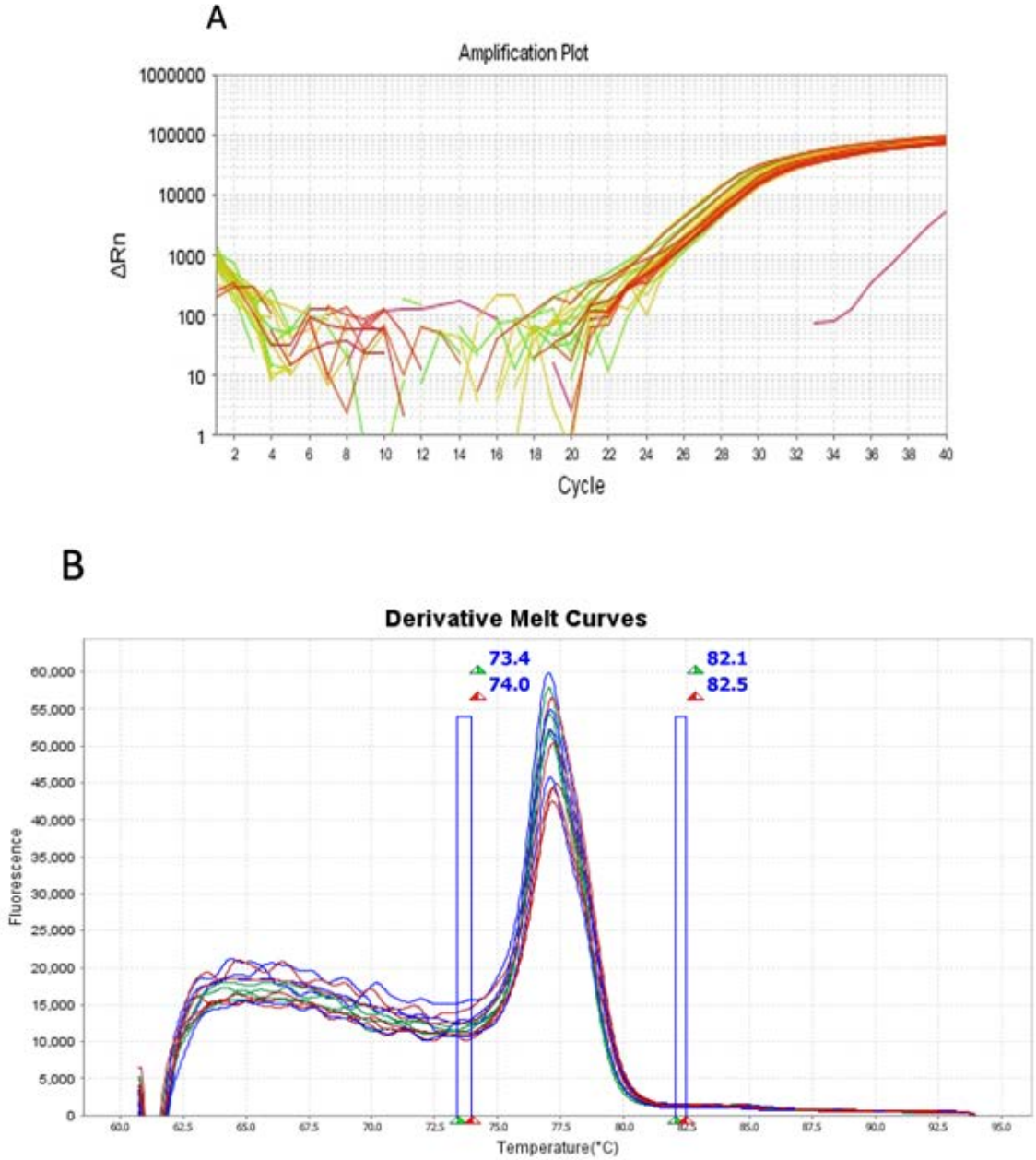

Figure 2. A) Amplification curve of the tested samples. B) Melting peak of the different PCR products

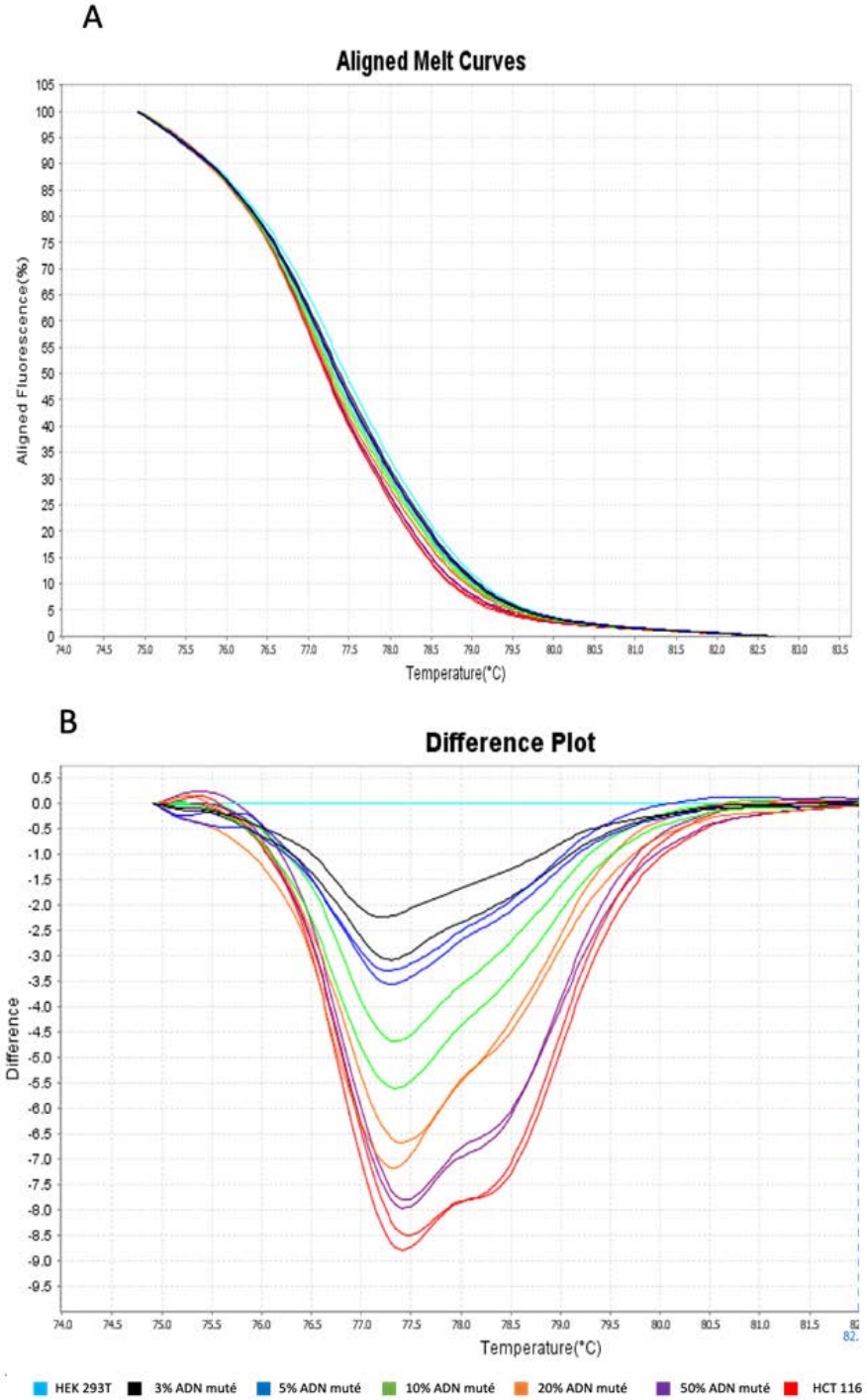

Figure 3. A) Melting curve of the different samples tested representing fluorescence as a function of temperature. B) Differential melting curve using the results obtained with the HEK293T line as reference. This curve represents the difference in fluorescence between the samples and the reference curve as a function of temperature

4. Discussion

The HRMA method is specific enough to discriminate a sample containing no mutated DNA from a sample containing only 3% mutated DNA. However, it was found that there was no significant difference between a sample containing 3% and a sample containing 5% mutated DNA. These results are in agreement with the recommendations of the National Cancer Institute (INCa) in France, which recommends in its September 2004 report on the validation of methods for the search for mutations in somatic genetics, to carry out the analyses on samples containing at least 5% of target DNA.

In the model used to evaluate the HRMA method, the study focused on the human genome, which is approximately 3400Mpb. It is therefore imperative to ensure through the melting curve of the real-time PCR that the primer pair used is specific to the region of interest.

This method has been widely used in industrialized countries such as Canada and France for research and diagnosis [12]. As has been shown, it is easy to implement in our context. The HRMA method is suitable for different types of genetic variation studies such as SNPs (Single Nucleotide Polymorphisms) detection, DNA methylation, mutation in patients, genotyping, and variant screening [13]. This method is also applicable in all fields of

research, microbiology (parasitology, bacteriology, virology, antimicrobial resistance) [14], human pathologies (oncology, immunology, reproduction), but also in plant fields [15].

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Conflict of Interest

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

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