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DETECTION OF *BRAF* MUTATION IN MELANOMA FOUND IN TISSUE AND CFDNA BY USING A HIGHLY SENSITIVE PNA-CLAMP PCR.

REGER R.MIKAEEL[*], ISAVELLA EVANGELOU, JOSHUA BILBIE,
JAMES HOWARD PRINGLE and GERALD SALDANHA[**]
[*]Dept. of Biology, College of Science, University of Duhok, Kurdistan Region-Iraq
[**] Dept. of cancer Studies and Molecular Medicine, University of Leicester-England

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ABSTRACT

Malignant melanoma results from ongoing activation of the mitogen activated protein kinases(MAPK) pathway, commonly driven by mutations in BRAF. Several selective inhibitors of this pathway are used clinically, most notably the Vemurafenib and Dabrafenib. Different methods for BRAF mutation detection exist in the United Kingdom, including pyrosequencing, COBAS test and Sanger sequencing. However, there is significant variability in the analytical sensitivity of these tests. A highly sensitive PCR assay was developed to detect low tumor cell percentage of 0.1% in formalin fixed paraffin embedded (FFPE) tissue and plasma. The assay was developed on stable cell lines containing BRAF (codon 600) mutations. Peptide nucleic acid (PNA) Clamp PCR was used for the selective amplification of DNA target sequences. Total DNA was extracted from 48 FFPE tissues and 20 blood plasma (14 matched) stage II-IV melanoma patients and screened for BRAF mutations. Results were correlated with COBAS test data and clinical parameters. PNA Clamp PCR on FFPE tissue showed 46% (n=22) BRAF mutants. Four more BRAF mutant cases were identified using PNA clamp methodology when compared to COBAS. Sample cases were independently tested. Matched samples showed 85% (n=12) correlation for BRAF mutation status (6 + ve, 4 – ve, 2 with no residual tumor burden). There were two cases which were positive for COBAS test and PNA Clamp PCR but negative for cfDNA. This demonstrates an innovative and highly sensitive technique for the detection of the common driver mutations in melanoma using exceptionally low tumor burden samples, representing a useful tool for future research and clinical application.

KEYWORDS: Melanoma, BRAF, Quantitative PCR, FFPE tissue, cfDNA

INTRODUCTION

Melanoma represents 4% of the common cancers (Cancer research uk. org, 2011), which counts as a significant disease burden worldwide and has major morbidity and mortality implications. The identification of common driver mutations in melanoma such as BRAF is the biggest step made in understanding melanoma so far. The RAF family comprises ARAF, BRAF and CRAF which are serine/threonine kinases. BRAF mutation is found in 43% of melanomas (Cancer. sanger. ac. uk, 2014). RAF is involved in MAPK pathway and in many cellular processes including cell proliferation, differentiation and transcriptional regulation. Mutations at codon 61 result in improved BRAF activity and increased phosphorylation of downstream targets. BRAF mutation in the majority of cases does not co-exist with any other mutation. BRAF is usually found in younger patients and have greater number of nevi. In comparison patients with chronic sun exposure were less likely to have BRAF mutations (Hacker et al., 2010). Vemurafenib and Dabrafenib inhibitors are reported to increase survival rate of melanoma patients. Vemurafenib was developed as a specific BRAF inhibitor for the V600E mutation. When patients are treated with BRAF inhibitors they show to have a temporary disease stabilisation and 15% of patients have a small progress at 6-8 weeks (Chapman P. et al., 2011). There are various methods for the detection of mutations in melanoma such as COBAS 4800 BRAF V600 test, Sanger sequencing, mutant allele-specific PCR, PNA clamp real-time PCR and pyrosequencing (Lyon E. et al., 2009). However, survival rates for BRAF inhibitors are poor and in relation to emerging resistance and how expensive the drugs are, new sensitive methods for detection

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of mutations and cost effective should be used. The need for a more sensitive and specific method for detecting mutations lead to the development and application of PNA Clamp PCR. According to Murdock et al., (2002), PNA Clamp PCR is a very sensitive method which can detect single nucleotide changes even if they exist in a small amount of the total DNA. As a result, where there is a wild- type and a mutant sequence, the wild-type can be blocked by the PNA and allow the amplification of mutant. PNA Clamp PCR can detect 1/20.000 alleles in some cases (Murdock D. et al., 2002). The high sensitivity of this method potentially will allow the detection of mutation in low frequency tumors and the treatment of more patients.

The cfDNA is the circulating free DNA present in the plasma or serum not only in cancer patients but also in healthy individuals. It is a double stranded molecule with molecular weight from 0.18-21 kB (Jahr S. et al., 2001, Stroun M. et al., 1987). However, the weight of cfDNA is significantly lower than genomic DNA (Jung K. et al., 2010). Research of cfDNA is poor due to nonspecific sensitive and analytical methods. Isolation procedures and sensitive PCR techniques with specific dyes have enabled progression in the research and now it is a potential diagnostic biomarker. In the past, detection of low concentration of cfDNA was not reliable (Kamm R. et al., 1972) in comparison to nowadays, with PCR assays and fluorescent dyes, cfDNA can be detected in healthy individuals as well. The aims and objectives of this project were to use a highly sensitive assay which can detect a low tumour cell percentage in BRAF by using cfDNA and FFPE tissues. PNA Clamp ASLNA qPCR was used for the selective amplification of DNA target sequences that differ by a single base pair. An assay for detecting BRAFV600E has already been developed and it was used to assess FFPE tissue samples and blood samples of melanoma patients to evaluate the frequency of BRAF mutations.

MATERIALS AND METHODS

Cell lines

Skin melanoma SK-MEL-28 and SK-MEL- 5 cell lines were used for this research. The two cell lines are malignant melanoma cell lines and were obtained from Dr. Pringle's lab (Reader in Molecular Pathology at University of Leicester) and cultured appropriately. SK-MEL-5 is derived from the skin and is heterozygous for BRAF p.V600E (c.1799T>A) and SK-MEL-28 is derived from the skin and is homozygous for BRAF p.V600E (c.1799T>A) (Cancer.sanger.ac.uk, 2014).

Melanoma samples

A number of 48 FFPE tissue blocks and 20 blood plasma of melanoma patients (14 matched with tissue) collected from 2008-2014; were obtained from pathology archives at Leicester Royal Infirmary and were tested for BRAF^{V600E} by PNA Clamp allele specific locked nucleic acid (ASLNA) quantitative PCR (qPCR) . All samples were taken from melanoma patients (2008-2014) and were tested for histological and clinic-pathological features. The clinic-pathological features of the melanoma samples are shown in table 1.

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Table (1): Clinic-pathological features of melanoma cases

Gender	
Male	35
Female	19
Age (median age 71 years)	
≤71 years	35
>71 years	19
Primary	33
Secondary	11
Unknown	4
Location	
Torso	22
Limbs	11
Head and neck	8
Acral melanoma	2
Mucosal melanoma	1
Unknown	4

Braf Mutation Analysis

Cobas 4800 Braf V600 Mutation Test

All FFPE tissue samples had been tested with COBAS 4800 BRAF V600 Mutation Test (Roche diagnostics), prior to quantitative PCR (qPCR) analysis. COBAS 4800 is able to detect BRAF V600 mutation in FFPE melanoma tissue. It is usually used in clinical trials to find the $BRAF^{V600E}$ status of the patients and help to decide which patients will receive BRAF inhibitor (vemurafenib) treatment. COBAS test is performed by using the DNA sample preparation kit according to the manufacturers' instructions.

Primer, Probe And Pna Design For Aslna Qpcr

BRAF ASLNA primers, probe and PNA for qPCR were designed and provided by previous students (Tayeb B. and Ehdode A., 2013). The sequences of *BRAF* primers, probes and PNA are listed in table 2.

The appropriate volume of sterile ultrapure (UP) water was added to the primer tubes to achieve concentration of 100 pmol/µl. Then, aliquots of primers were made by diluting 5 µl of the primer stock in 95 µl of UP water (1:10 dilution). Peptide nucleic acid (PNA) was diluted in the same manner as primers. Probe aliquots were made by diluting 2 µl of probe in 98 µl of UP water (1:50 dilution).

Table (2): ASLNAqPCR primers, probes and PNA for $BRAF^{V600E}$

Forward primer +LNA	Wild type- TAGGTGATTTTGGTCTAGCTACAG+T
	Mutant- TAGGTGATTTTGGTCTAGCTACAG+A
Reverse primer	ATCCAGACAACTGTTCAAACTGATG
Probe	Fam-AATCTCGATGGAGTGGGT-MGB
PNA	TAGCTACAGTGAAATC

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Cell culture

The cell lines that were cultured were SK-MEL-28 and SK-MEL- 5. At first, cells were resuscitated from liquid nitrogen and defrosted at 37^{O}C in water bath (Grand Instruments, Cambridge). The cells were transferred to a sterile falcon tube (Fisher Scientific, Loughborough, UK) containing 10 mL pre-warmed Roswell Park Memorial Institute – 1640 (RPMI-1640) (Lonza, Verviers, Belgium) with 10% foetal calf serum (FCS) (Sera-lab) and centrifuged (Scientific Laboratory Supplies) at 1000 rpm for 5 minutes. Media was removed and the cell pellet was re-suspended in pre-warmed RPMI-1640 media, transferred to a 25 mL dewar flask (T-25) (Sigma-Aldrich) in proportion 1:5, and incubated overnight (o/n) at 37^{O}C in 5% CO2 atmosphere. The following day growth media was removed and the cells monolayer was washed twice with pre-warmed (37^{O}C) phosphate buffered saline (PBS) (Lonza. Then, 5 mL of pre-warmed trypsin with Ethylenediaminetetraacetic acid (EDTA) solution were added on the cells. Cells were then centrifuged with an equal amount of growth medium (the growth medium neutralised the trypsin). Cell pellets were re-suspended in pre-warmed growth media and allowed to grow until they reach 80-90% confluence.

For DNA extraction, the same procedure was followed but cells after re-suspension they were divided into 1.5 mL tubes and centrifuged at 14000 rpm for 5 minutes. A pellet was formed and the growth media was discarded. Cell pellets were stored at -80^{O}C for DNA extraction.

DNA extraction from cell lines

DNA was extracted from SK-MEL-28 and SK-MEL- 5 cell lines cell. Cell pellets of each cell line were re-suspended in 250 µl of 0.05M Tris pH 8/0.1% SDS. Proteinase kinase (PK) was then added. After incubation an equal amount of phenol/chloroform/IAA was then added to the tube and centrifuged. The top layer (aqueous) of the solution was added to a clean 1.5 mL tube and an equal amount of phenol/chloroform/IAA (Sigma Aldrich) was added and centrifuged. The top layer was again transferred to a clean tube. Cold ethanol (-20^{O}C) and 1M sodium chloride (NaCl) ($^{1}/_{10}$ of starting volume; 2.5 µl) were added to the mixture and left at -20^{O}C for 30 minutes and microfuged at 4^{O}C. Following, the pellet was washed with ethanol and centrifuged and the ethanol was left to air dry. Finally the pellet was re-suspended in ultrapure sterile water and stored at 4^{O}C.

DNA extraction from FFPE tissue

FFPE tissues were prepared and the DNA was extracted using the QIAamp DNA FFPE tissue kit (Qiagen, Hilden, Germany; cat.no: 56404). Slides were incubated at 65^{O}C for 10 minutes and then they were de- waxed and rehydrated. ATL buffer were used to scrape the sections and 10 µl of proteinase K recombinant were added and incubated. Then, 200 µl AL buffer were added in each tube, centrifuged and transferred safe-lock eppendorfs. Samples were heated and transferred to 1.5 mL tubes. 200 µl of absolute ethanol were added and centrifuged. Samples were then applied on QIAamp columns and centrifuged. Columns were then re-placed in a new collection tube and 500 µl buffer AW1 were added and centrifuged again as before. Then, 500 µl of buffer AW2 were added and centrifuged. After that, 35 µl buffer AE were added. Elution of DNA was transferred to a new tube and stored at 4^{O}C.

cfDNA extraction from blood plasma

Circulating cell-free (cfDNA) was extracted from blood plasma using QIAamp Circulating Nucleic acid kit (Qiagen, Hilden, Germany). Plasma samples (1 mL) were thawed and centrifuged and 100 µl PK (Qiagen, Hilden, Germany) was added and 1 mL of plasma supernatant was added. 0.8 µl of ACL buffer were added, vortex for 30 seconds and incubated at water bath.

An additional 1.8µl of ACB buffer were added, vortex for 30 seconds and incubated on ice. 680 µl of the mixture were added to the QIAamp columns each time and centrifuged. Then, ACW1 buffer were added and centrifuged. The same procedure was repeated for buffer ACW2 (700 µl) centrifuged at 14000 rpm for 3 minutes and incubated. Then, 50 µl of AVE buffer were applied and incubated at RT for 3 minutes and were centrifuged.

Real time PCR

Quantitative real-time polymerase chain reaction (qPCR) for BRAF gene was performed. ASLNAqPCR was completed by using 7 µl of mastermix which included 5 µl of TaqMan Genotyping Master Mix (Applied Biosystems, cat.no: 4371355, Cheshire, UK), 0.2 µl of appropriate forward primer, reverse primer, probe

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and PNA and the remaining amount of µl was used for sterile UP water. An additional 3 µl (10 ng/3 µl) of DNA was added to each well to give a final amount of 10 µl. The positive controls that were used for qPCR were human genomic DNA (HGD) and cell lines and for negative control no template control/sterile UP water (NTC) was used. ΔCt was collected from all samples after 50 cycles.

Ct and ΔCt

The cycle threshold (Ct) is the amount of cycles needed for the signal to be produced. If the amount of target nucleic acid**,** in our case DNA**,** is low the Ct value will be higher and vice versa. For a qPCR of 40 cycles the following rules define the amount of DNA in the samples. If:

a) Cts < 29: strong positive reactions, large amount of DNA in the sample

b) Cts of 30-37: are positive reactions, moderate amounts of DNA in the sample.

c) Cts of 38-40: are weak reactions, minimal amounts of DNA which can occur by contamination.

However, we used 50 cycles as we were using touchdown (TD) method that increases the number of cycles by 10-15.

Statistical analysis

Microsoft Excel (2013) and GraphPad Prism version 6 (GraphPad software, California, United States) software were used for statistical analysis.

RESULTS

In order to find the frequency of mutations in melanoma, Catalogue of Somatic Mutations in Cancer (COSMIC) was used. The location of mutations in BRAF in figure 1.

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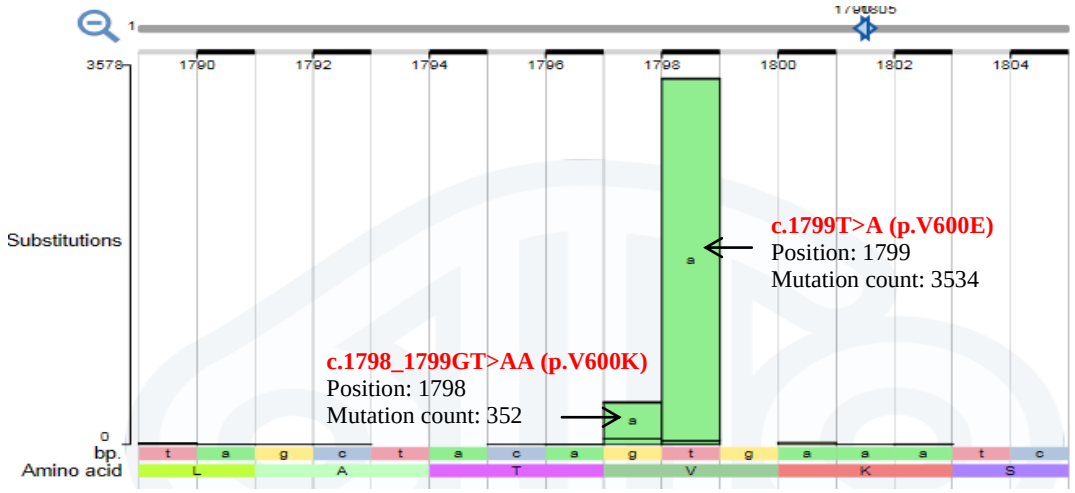

Fig (1): Location of BRAF mutations according to COSMIC software. The most common mutation for BRAF appears to be at location c.1799 T>A (p.V600E).

The frequency of each mutation in malignant melanoma was found to be 43% for *BRAF* mutations of which ~90% of them was V600E.

3.4 Specificity of BRAF^{V600E} primers for PNA Clamp ASLNAqPCR

BRAF^{V600E} LNA primers and PNA for ASLNAqPCR were designed by previous students (Tayeb B. and Ehdode A., 2013). However, we checked the primers with HGDNA and cell lines (Figure 2). For HGDNA, wild-type LNA/-PNA had a Ct of 24 cycles, wild-type LNA/+PNA had a Ct of 32 cycles which gives a ΔCt of 8 cycles. The mutant LNA/±PNA did not amplify. For SK-MEL-5 mutant cell line, mutant LNA/±PNA had a Ct of 24 cycles, wild-type LNA/-PNA 26 cycles and wild-type LNA/+PNA 35 cycles (Figure 2).

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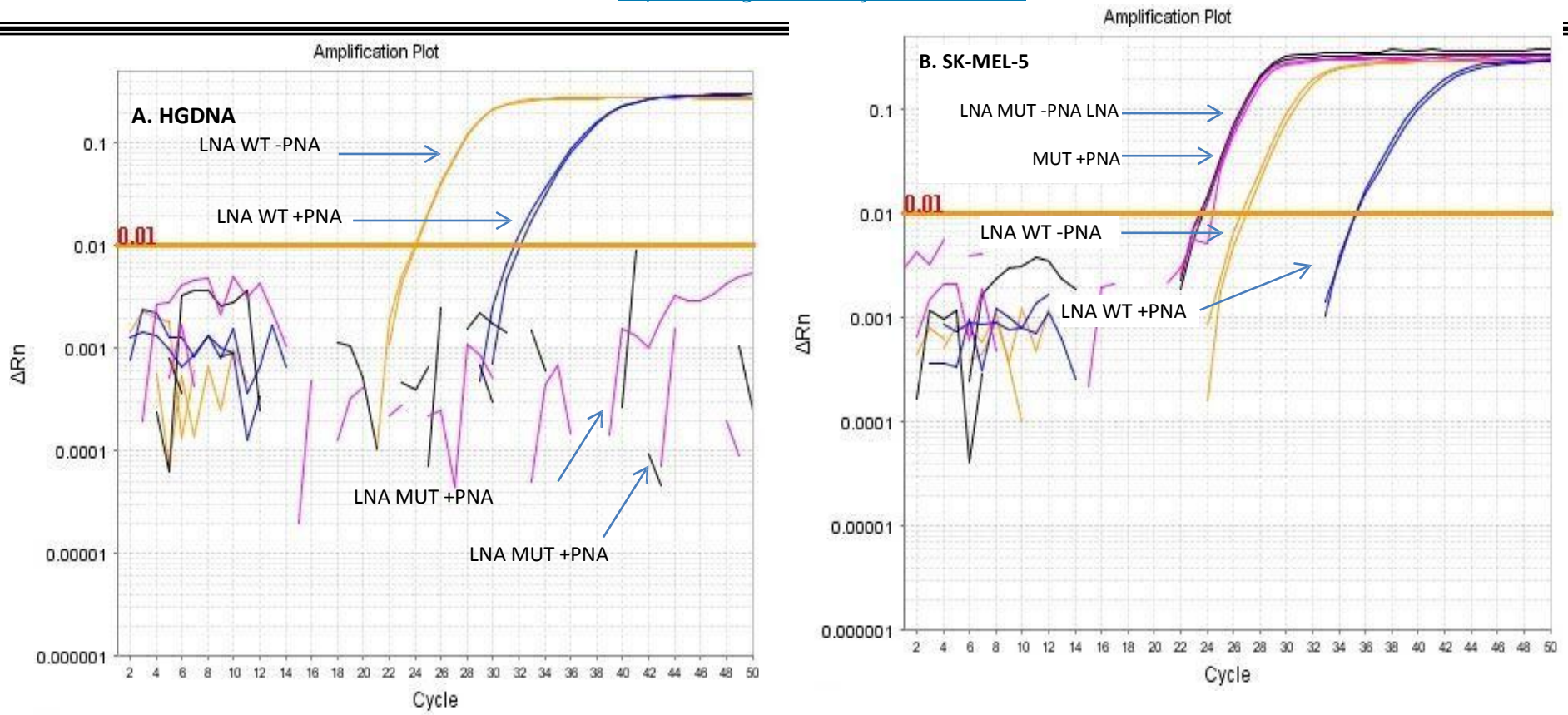

Fig. 2: Specificity of BRAFV600E primers for PNA Clamp QUASAqPCR. Amplification plots of LNA wild-type and mutant primers ±PNA for HGDNA and SK-MEL-5 cell line (LNA wild-type primer –PNA: orange, LNA wild-type primer +PNA: blue, LNA mutant primer –PNA: black, LNA mutant primer +PNA: pink).

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7. Analyses of clinical Samples

The nature of the specimens varied. The 65% of the cases were men and the 35% women with median age of 71 years old; 65% of them were younger and 35% older than 71 years old.

/3.7.1 BRAF^{V600E} PNA Clamp ASLNAqPCR

PNA Clamp ASLNAqPCR assay was used to detect BRAF^{V600E} mutation. For FFPE samples the ΔCt for SK-MEL-28 and SK-MEL-5 mutant cell lines was -3 and 0.5 cycles respectively. HGDNA had a ΔCt more than 20 cycles as there was no amplification, PNA completely supressed the wild-type. Negative control also did not amplify. With the wild-type primer HGDNA had a Ct of 28 cycles. Samples that amplified for BRAF^{V600E} were considered as mutated (Figure 3 A) and samples that did not amplify were considered as wild type (Figure 3 B).

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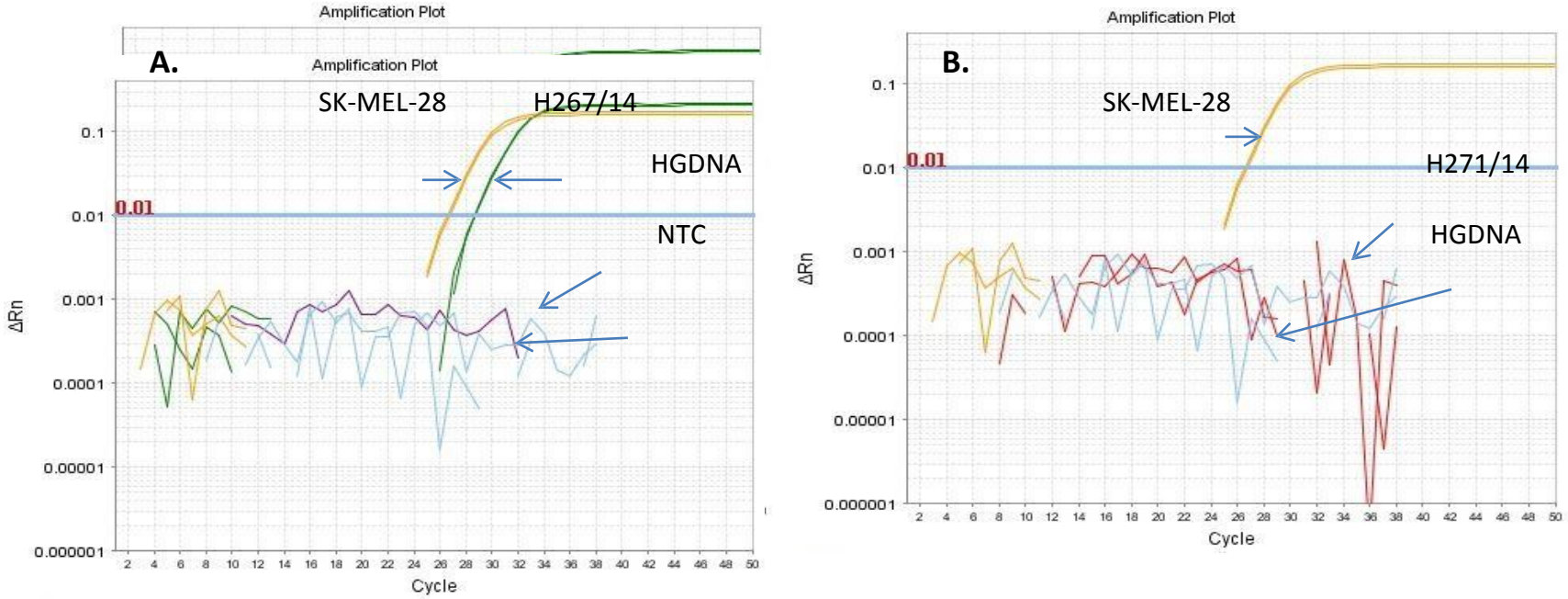

Fig. 3: Amplification plots of FFPE samples using PNA Clamp ASLNAqPCR. A) Representation of the melanoma cell line SK- MEL-28 amplification (orange) with a ΔCt of 27 cycles and a mutated for $BRAF^{V600E}$ sample H267/14 (green) with a ΔCt of 29 cycles. HGDNA (blue) did not amplify along with the NTC (purple). B) Representation of the melanoma cell line SK-MEL-28 amplification (orange) with a ΔCt of 27 cycles and a wild -type for $BRAF^{V600E}$ sample H271/14 (red). HGDNA (blue) did not amplify along with the NTC (purple).

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For cfDNA samples the ΔCt for SK-MEL-28 and SK-MEL-5 mutant cell lines was -4 and 0.3 cycles respectively. HGDNA had a ΔCt more than 20 cycles as there was no amplification, PNA completely supressed the wild-type. Negative control also did not amplify. With the wild-type primer HGDNA had a Ct of 23 cycles. Samples that amplified for $BRAF^{V600E}$ were considered as mutated (Figure 4A) and samples

that did not amplify were considered as wild type (Figure 4B).

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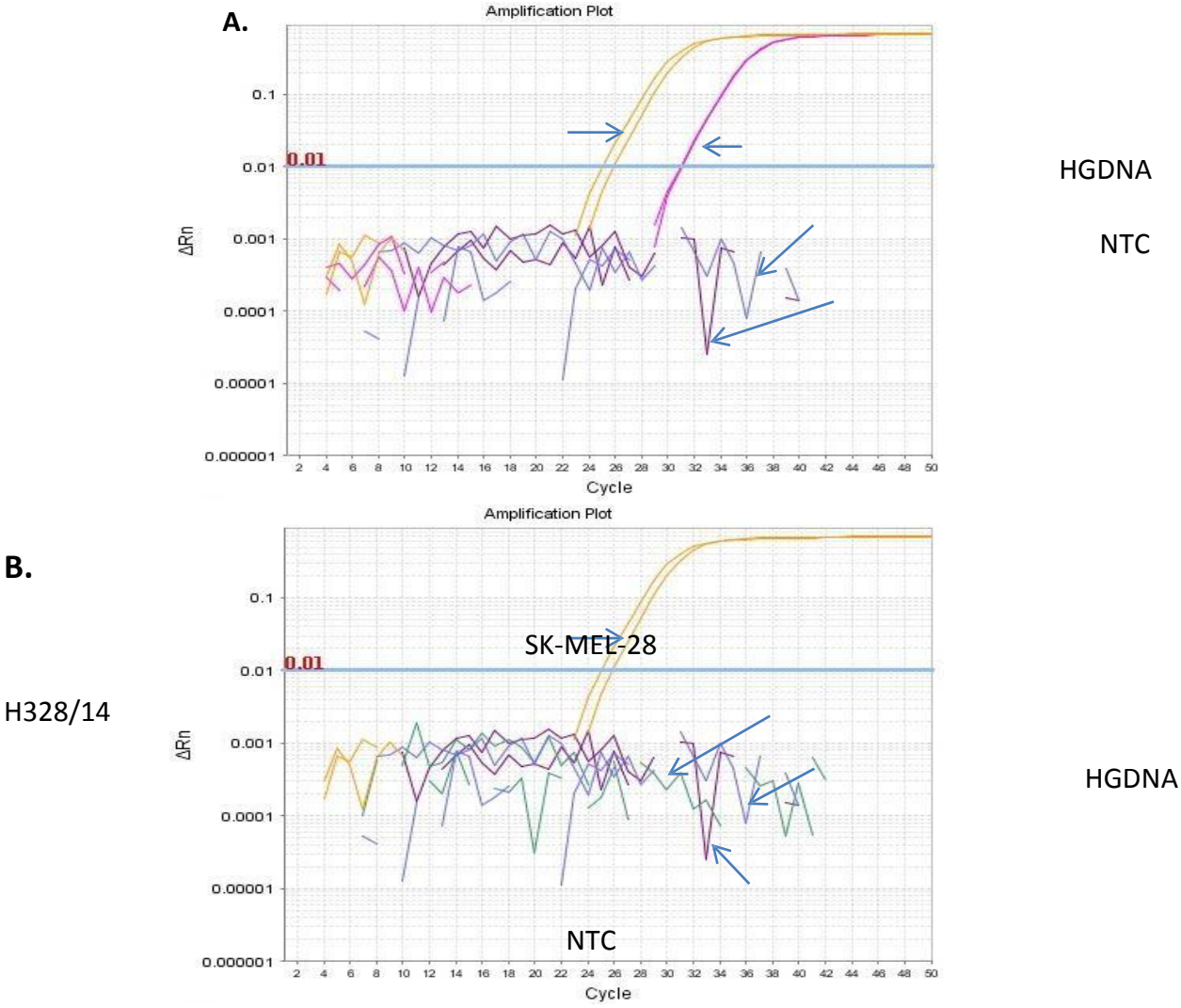

Fig. 4: Amplification plots of cfDNA samples using QUASAqPCR. A) Representation of the melanoma cell line SK-MEL-28 amplification (orange) with a ΔCt of 26 cycles and a mutated for $BRAF^{V600E}$ sample H631/13 (pink) with a ΔCt of 31 cycles. HGDNA (blue) did not amplify along with the NTC (purple). B) Representation of the melanoma cell line SK-MEL-28 amplification (orange) with a ΔCt of 26 cycles and a wild -type for $BRAF^{V600E}$ sample H328/14 (green). HGDNA (blue) did not amplify along with the NTC (purple).

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The status of the cases was confirmed by plotting the ΔCt (y axis) against the wild-type

Ct (x axis). Cases with undetermined mutant Ct were given a mutant Ct mean value of 50 cycles in order to calculate the ΔCt. Negative samples for $BRAF^{V600E}$ had a ΔCt >10 cycles. A ‘’cut off’’ line was drawn to represent the detectable amount of DNA and determine the status of samples that had single amplification. The line started approximately 10 cycles after the cell line amplification cycle and was parallel to the line formed by samples that appeared to be wild-type. Samples below the line were considered as mutated for the $BRAF^{V600E}$ mutation and samples above the line considered as wild-type tumours for $BRAF^{V600E}$. Samples very close to the ‘’cut-off’’ line were considered as wild-type. PNA Clamp ASLNAqPCR detected $BRAF^{V600E}$ in 22 out of 48 corresponding to 46% of the samples (Figure 5 A). 21 out of 22 mutated FFPE samples amplified in duplicate (pink) and 1 had single amplification (turquoise). The information for mutated FFPE samples is shown in table (5) and extended information in the appendix. cfDNA samples were also tested for the $BRAF^{V600E}$ mutation. PNA Clamp ASLNAqPCR detected $BRAF^{V600E}$ in 8 out of 20 corresponding to 40% of the samples (Figure 5 B). 6 out of 8 mutated cfDNA samples amplified in duplicate and 2 had single amplification. The information for mutated cfDNA samples is shown in table (6) and extended information in the appendix. For six of the cfDNA samples matched tissue wasn’t available and two of them were mutated for $BRAF^{V600E}$. 10 of the remaining 14 matched samples were mutated for $BRAF^{V600E}$ on FFPE tissue and 6 of them mutated for $BRAF^{V600E}$ on cfDNA.

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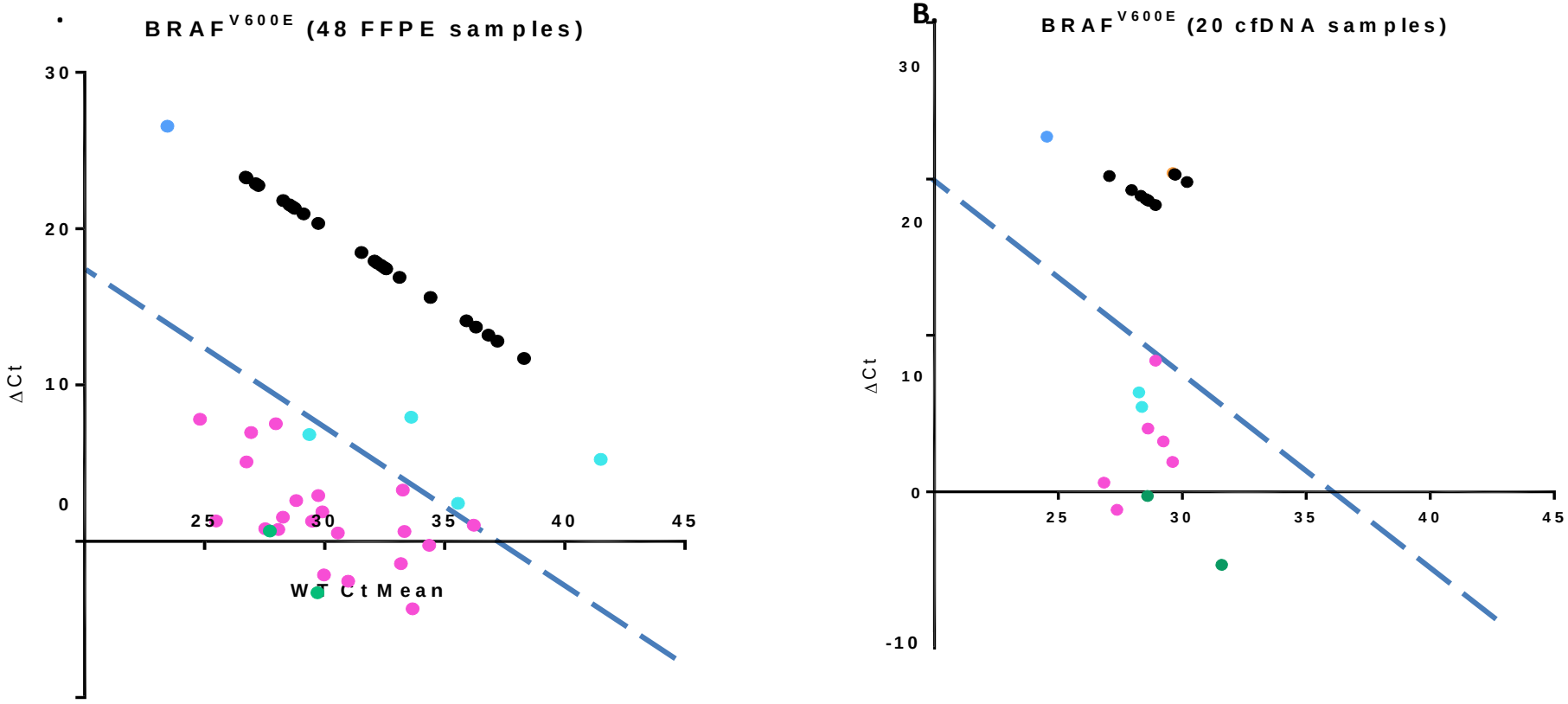

Fig. 5: Plots of BRAF^{V600E} PNA Clamp ASLNAqPCR mutated and wild-type cases for FFPE (n=48) and cfDNA (n=20) samples. Lines represent the ‘’cut off’’ detectable amount of DNA and determine the status of samples that had single amplification. Line starts ≈10 cycles after cell line Ct mean cycle. Samples below the line were mutated (pink) and samples above the line are wild-type (black). (FFPE samples: 22 (46%) mutated and 26 (54%) wild-type; cfDNA samples: 8 mutated (40%), 12 wild-type (60%); blue: HGDNA; green: cell lines).

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Table (5): Mutated FFPE samples for BRAF^{V600E} PNA Clamp ASLNAqPCR

FFPE Block no	WT Ct	MUT Ct	ΔCt
H244/14	27.529	28.332	0.803
H245/14	36.21	37.24	1.03
H247/14	33.249	36.52	3.271
H252/14	29.974	27.817	-2.156
H255/14	33.174	31.739	-1.435
H257/14	34.346	34.087	-0.259
H258/14	33.317	33.937	0.62
H263/14	33.662	29.338	-4.324
H264/14	30.979	28.426	-2.553
H267/14	28.256	29.803	1.547
H269/14	28.816	31.418	2.602
H272/14	24.812	32.62	7.808
H274/14	29.363	36.192	6.829
H275/14	25.479	26.78	1.301
H278/14	29.729	32.659	2.93
H398/14	29.906	31.785	1.878
H399/14	30.545	31.071	0.526
H403/14	27.968	35.486	7.518
H404/14	28.067	28.825	0.758
H406/14	26.742	31.822	5.08
H407/14	29.473	30.756	1.284
H409/14	26.938	33.889	6.951

Table (6): Mutated cfDNA samples for BRAF^{V600E} PNA Clamp ASLNAqPCR

cfDNA no	Matched FFPE	WT Ct	MUT Ct	ΔCt
H573/13	H244/14	29.613	31.523	1.909
H631/13	H272/14	29.234	32.457	3.223

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H337/14	H399/14	28.254	34.607	6.352
H492/13	H404/14	28.368	33.798	5.43
707/12	H407/14	27.362	26.208	-1.154
H348/14	H409/14	28.915	37.299	8.384
H391/14	-	28.611	32.661	4.05
H392/14	-	26.841	27.43	0.589

Comparison of COBAS 4800 BRAF V600 mutation test and PNA Clamp PCR for FFPE tissue samples

COBAS 4800 test and PNA Clamp PCR were used on FPPE tissue samples to identify the *BRAFV600* status of the samples. By comparing these methods, it was found that 40% of the samples (n=19) were mutated for both methods, 0% were wild-type with PNA Clamp PCR and mutated with COBAS 4800 test, 8% (n=4) were mutated for PNA Clamp PCR but wild-type for COBAS 4800 test and 52% (n=25) were wild-type for both methods (Table 7). According to this comparison, the COBAS test was 100% sensitive, 86% specific and 92% accurate.

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Table 7: Comparison of COBAS 4800 *BRAF V600* mutation test and PNA Clamp PCR for FFPE tissue samples.

PNA Clamp PCR / COBAS 4800	*BRAF*^{V600E} mutated	*BRAF*^{V600E} wild-type
BRAF^{V600E} mutated	19	0
BRAF^{V600E} wild-type	4	25

$$\textbf{Sensitivity} = \frac{qPCR/COBAS + ve}{(qPCR/COBAS + ve) + (qPCR - ve/COBAS + ve)} = \frac{19}{19} = 1 = \mathbf{100}\%$$

$$\textbf{Specificity} = \frac{\text{qPCR} - \text{ve/COBAS} - \text{ve}}{(\text{qPCR} - \text{ve/COBAS} - \text{ve}) + (\text{qPCR} + \text{ve} \,/\text{COBAS} - \text{ve})} = \frac{25}{29} = 0.86 = \mathbf{86}\%$$

$$\textbf{Accuracy} = \frac{\left(\frac{\text{qPCR}}{\text{COBAS}} + \text{ve}\right) + \left(\text{qPCR} - \frac{\text{ve}}{\text{COBAS}} - \text{ve}\right)}{\text{Total number of cases}} = \frac{44}{48} = 0.916 = \mathbf{92}\%$$

3.9 Patients stage and concentration of cfDNA

The concentration of cfNDA varied from 0.003-0.533ng/3µL. The patients' stage ranged from stage II-IV; fifteen samples were stage IV, two stage III, one stage II and two patients that had tumour excision. The information for the stage and cfDNA concentration of each samples in shown in figure 6.

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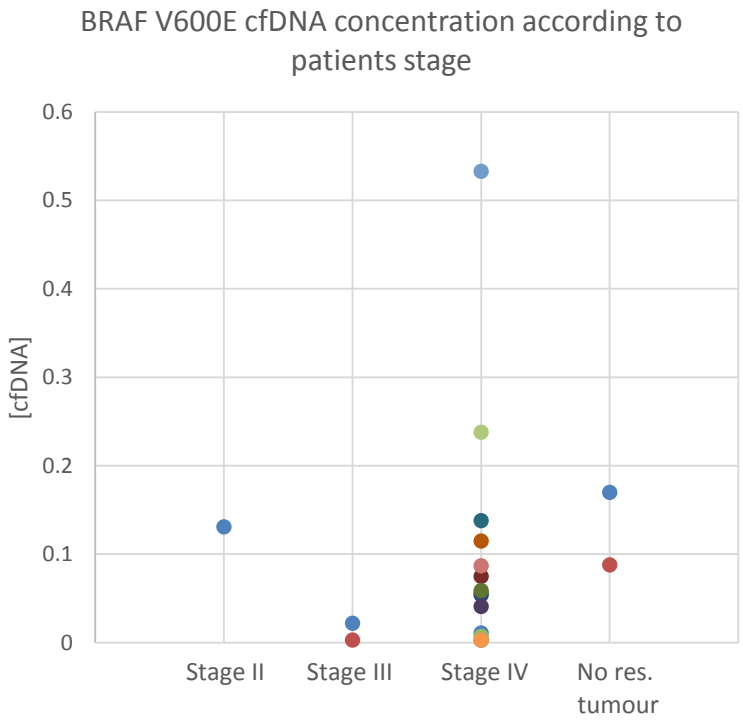

Fig. (6): Patient's stage and concentration of cfDNA

3.10 Information on clinical samples

A number of 14 cfDNA samples were available that matched FFPE tissue that were already analysed with qPCR (6 *BRAF* FFPE and cfDNA +v and 4 *BRAF* FFPE +ve). Information for the cases is shown in table 8.

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Table (8): *Matched FFPE tissue with cfDNA samples*

FFPE Block no.	cfDNA no.	COBAS	FFPE Tissue	cfDNA*	Comments
			BRAF	**BRAF**	
H242/14	H347/14	Negative	-ve	-ve	!!
H244/14	H573/13	Positive	+ve	+ve	-
H249/14	H493/14	Negative	-ve	-ve	!!
H251/14	H668/13	Negative	-ve	-ve	!!
H272/14	H631/13	Positive	+ve	+ve	-
H275/14	H327/14	Positive	+ve	-ve	Previous metastatic disease, but all metastases excised. No residual tumour.
H398/14	H338/14	Positive	+ve	-ve	Previous BRAF inhibitor
H399/14	H337/14	Positive	+ve	+ve	Previous BRAF inhibitor stopped due to toxicity
H400/14	H378/14	Negative	-ve	-ve	!!
H403/14	H1436/14	Positive	+ve	-ve	no information about treatment
H404/14	H492/13	Positive	+ve	+ve	BRAF inhibitor after blood taken
H406/14	H669/14	Positive	+ve	-ve	Previous metastatic disease, but all metastases excised. No residual tumour.
H407/14	H1707/14	Positive	+ve	+ve	!!
H409/14	H348/14	Positive	+ve	+ve	!!
Total: 14 cases (6 BRAF FFPE and cfDNA and ve, 4 BRAF FFPE)					

*FFPE tissue and cfDNA taken at different time points. !!: metastatic disease but no treatment (if BRAF +ve) but may have had treatment (if BRAF –ve but obviously not a BRAFi e.g. dacarbazine or ipilumimab)

DISCUSSION

Testing of tumor tissue remains the recommended method for detecting the presence of somatic mutations in human malignancies. Melanoma is one of the most aggressive forms of cancers and targeted therapy for driver mutations like BRAF is essential (Molina et al., 2015). BRAF mutation is found in 43% of melanomas (Cancer.sanger.ac.uk, 2014) and ~90% of these are thymine to adenine substitutions at codon 600 valine to glutamine at position c.1799T>A (V600E). Therefore, V600E is considered to be the most frequent somatic point mutation in metastatic melanoma, providing a unique molecular marker for this malignancy (Lovly et al., 2012 and Rubinstein et al., 2010). In this project we evaluated whether the frequency of detected BRAFV600E mutations in melanoma and whether cfDNA can be used as a prognostic factor for melanoma patients. For this reason, a BRAFV600E mutation detection assay was developed based on Real time PCR and specifically the technique applied was PNA Clamp ASLNA qPCR. Results were compared with COBAS 4800 BRAFV600E mutation test, which is the only test approved for selection of patients for treatment with BRAF inhibitors based on positive mutation test.

ASLNAqPCR use primers with an extra LNA base on their 3'-end. An ASLNA assay is cost effective, very sensitive and specific and also gives information about the ratio of wild-type and mutant alleles (Morandi L. et al., 2012). In our study, LNA was used to increase PCR sensitivity. LNA's have been proved to be useful for mutation detection with high sensitivity for KRAS and BRAF (Arcila M. et al., 2011). All the samples were analysed by PNA Clamp ASLNAqPCR and the status of each sample was determined by the amplification of each sample. Samples were determined as mutant when tested with mutant primer and there was amplification in duplicate. If there was no amplification samples were considered as wild type, negative for

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BRAFV600E mutation. For some of the samples the status was not clear as there was single amplification. In order to overcome this problem the status of the cases was confirmed by plotting the wild-type Ct (x axis) against the ΔCt (y axis). Cases with undetermined mutant Ct were given a mean value of 50 cycles in order to calculate the ΔCt. Negative samples for BRAFV600E had a ΔCt >10 cycles. A "cut off" line, the slope, showing the single genome amplification, defining the theoretical limits for detecting mutant alleles (1.6 molecules) was drawn to represent the detectable amount of DNA and determine the status of samples that had single amplification. The line started approximately 10 cycles after the cell line amplification cycle and was parallel to the line formed by samples that appeared to be wild-type for BRAFV600E. Samples below the line were considered as mutated for the BRAFV600E mutation and samples above the line considered as wild-type tumours for BRAFV600E. Samples very close to the "cut-off" line were considered as wild-type. In a study of 193 patients, 48% of the cases harbour BRAF mutations of which 71% were V600E (Carlino M. et al., 2014). In comparison Sclafani et al. (2013), found that BRAF mutations in melanoma are up to 66% in exons 11 and 15 but the majority of the mutations were at position 1799 T>A and only few belong to exon 11 (Sclafani F. et al., 2013). The research in melanoma has been improved by the advances in BRAF inhibitors. The use of the BRAF inhibitors to treat patients that hold BRAFV600E mutation results to the reduction of advanced lesions (Weeraratna A., 2012). The first response to vemurafenib is very impressive with success rate of ≈ 50 % and also increased survival however, resistance to the inhibitor starts after 2-18 months after the first treatment (Cox A. et al., 2012). Moreover, secondary tumours may occur from the BRAF inhibition such as cutaneous squamous-cell carcinomas and keratoacanthomas (Weeraratna A., 2012). In addition by comparing the COBAS 4800 BRAFV600E test with PNA Clamp ASLNAqPCR on FFPE tissue samples, results show that the biological sensitivity of the assay was 100% as all the samples that were BRAF mutated with COBAS test were also mutated for PNA Clamp ASLNAqPCR. The biological specificity was 86% and the accuracy was 92%. These results suggest that PNA Clamp ASLNAqPCR assay was more sensitive than the COBAS 4800 test as it detected four positive samples that were not detected with the COBAS test. The use of FFPE tissue from biopsies is essential for immunohistochemistry and haematoxylin and eosin staining but has several arising issues when is used with molecular methods such as qPCR. First of all, cross-linking is caused by formaldehyde which covalently links nucleic acids resulting is lower efficiency of molecules in qPCR (Perkel, 2014). Moreover, in cancer patients when the position of the tumour is difficult to perform biopsy, for example in lung cancer patients, the tumour material is inadequate (Sequist L. et al., 2009). In addition, the amount of tumour in FFPE tissue is low as it is mixed with normal tissue (Plesec T. et al., 2009). According to Fleischhacker et al. (2008) and Gerlinger et al., (2012), biopsies are taken from a part of the tumour arising the issue if all the mutant clones are included in tumour heterogeneity; leading to the conclusion that the specificity and sensitivity of qPCR is greatly affected as there are chemical changes in DNA and low number of molecules, resulting in higher Ct signalling Fleischhacker et al. (2008) and Gerlinger et al., (2012). On the other hand, cfDNA in qPCR might be a better biomarker as it is not formalin fixed and less non-specific binding should be observed resulting in an increase of the sensitivity and the specificity (Aung K. et al., 2014).

According to a study by Board et al. (2008), cfDNA can be extracted by both plasma and serum with serum having the disadvantage of white blood cells (Board R. et al., 2008). In our study, BRAFV600E ASLNAqPCR assay showed a mutant ratio of 1:1000 mutant to wild type DNA. Fourteen cases that had matched tissue available were tested in both FFPE tissue and cfDNA in two different time points; FFPE tissue was tested first and then blood sample was taken at later time. Six of the cases were BRAF positive in both FFPE and cfDNA, four cases were BRAF positive only in FFPE tissue, one case was NRAS Q61R positive in FFPE tissue and three cases were negative. There is an explanation for mutation status in FFPE tissue and cfDNA samples. For the samples that were BRAFV600E positive for both FFPE and cfDNA, three had metastatic disease but did not receive treatment (H492/13 / H404/14, H1707/14 /H407/14 and H348/14 / H409/14), one was treated BRAF inhibitor but the treatment stopped due to toxicity (H399/14 / H337/14) and for two of the cases no clinical information for any treatment was given

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(H244/14 / H573/13 andH272/14 / H631/13). For the four cases that were BRAFV600E positive in FFPE tissue analysis, for two of them tumour was excised (no residual tumour) (H275/14 / H327/14 and H406/14 / H669/14), one was treated with BRAF inhibitor (H398/14 / H335/14) and for one no information was available (H403/14 /H1436/14). One case was positive for NRAS Q61R on FFPE tissue analysis and three other cases were negative for BRAFV600E which had treatment with non BRAF inhibitor (e.g dacarbazine or iptumimab). As a result it could be argued that the sensitivity of ASLNAqPCR and QUASAqPCR methods to detect mutation can be 100%.

The use of cfDNA mutation testing could be used as an initial screening step to determine the patients with BRAF mutant tumours for clinical trials which will be very beneficial as more patients with metastatic disease will be identified earlier which will therefore improve efficiency of future clinical trials. In addition this method will be very useful in cases where biopsy is difficult to be performed.

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APPENDIX

Table (9): *BRAF* codon 600 V600E c.1799 T>A PNA Clamp ASLNAqPCR information for FFPE samples.

Sample	WT Ct Mean	MUT Ct Mean	ΔCt	Status
H238/14	41.499	46.740	5.241	1
H239/14	35.546	37.967	2.421	1
H240/14	38.304	50	11.696	
H241/14	36.818	50	13.182	
H242/14	27.242	50	22.758	
H243/14	34.403	50	15.597	
H244/14	27.529	28.332	0.803	2
H245/14	36.210	37.240	1.030	2
H246/14	37.194	50	12.806	
H247/14	33.249	36.520	3.271	2
H248/14	36.294	50	13.706	
H249/14	27.129	50	22.871	
H250/14	32.567	50	17.433	
H251/14	26.747	50	23.253	
H252/14	29.974	27.817	-2.156	2
H253/14	32.348	50	17.652	
H254/14	32.073	50	17.927	
H255/14	33.174	31.739	-1.435	2
H256/14	32.486	50	17.514	
H257/14	34.346	34.087	-0.259	2
H258/14	33.317	33.937	0.620	2
H259/14	33.605	41.547	7.942	1
H260/14	31.527	50	18.473	
H261/14	33.117	50	16.883	
H262/14	35.903	50	14.097	
H263/14	33.662	29.338	-4.324	2
H264/14	30.979	28.426	-2.553	2
H266/14	28.532	50	21.468	
H267/14	28.256	29.803	1.547	2
H268/14	28.760	50	21.240	
H269/14	28.816	31.418	2.602	2
H270/14	29.121	50	20.879	
H271/14	29.730	50	20.270	
H272/14	24.812	32.620	7.808	2
H273/14	32.178	50	17.822	
H274/14	29.363	36.192	6.829	1
H275/14	25.479	26.780	1.301	2
H276/14	26.710	50	23.290	

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H277/14	28.669	50	21.331	
H278/14	29.729	32.659	2.930	2
H398/14	29.906	31.785	1.878	2
H399/14	30.545	31.071	0.526	2
H400/14	28.269	50	21.731	
H403/14	27.968	35.486	7.518	2
H404/14	28.067	28.825	0.758	2
H406/14	26.742	31.822	5.080	2
H407/14	29.473	30.756	1.284	2
H409/14	26.938	33.889	6.951	2

Table(10): *BRAF* codon 600 V600E c.1799 T>A PNA Clamp ASLNAqPCR information for cfDNA samples.

Sample	WT Ct Mean	MUT Ct Mean	ΔCt	Status
H347/14	29.614	50	20.386	
H573/13	29.614	31.523	1.909	2
H493/14	28.532	50	21.468	
H668/13	28.604	50	21.396	
H631/13	29.234	32.457	3.223	2
H327/14	28.634	50	21.366	
H338/14	29.691	50	20.309	
H337/14	28.254	34.607	6.352	2
H378/14	30.192	50	19.808	
H1436/12	27.953	50	22.047	
H492/13	28.368	33.798	5.430	2
H669/13	29.720	50	20.280	
H1707/12	27.362	26.208	-1.154	2
H348/14	28.915	37.299	8.384	2
H336/14	28.916	50	21.084	
H328/14	27.056	50	22.944	
H346/14	29.603	50	20.397	
H335/14	28.329	50	21.671	
H391/14	28.611	32.661	4.050	2
H392/14	26.841	27.430	0.589	2