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BRAF V600E Mutation Detection Using High Resolution Probe Melting Analysis

Jennifer E. Hardingham[1,2], Ann Chua[1], Joseph W. Wrin[1], Aravind Shivasami[1], Irene Kanter[1], Niall C. Tebbutt[3] and Timothy J. Price[1,2]
[1]The Queen Elizabeth Hospital, Adelaide, SA, 5011
[2]University of Adelaide, SA, 5005
[3]Ludwig Institute for Cancer Research, Austin Health, Melbourne, VIC, 3084
Australia

1. Introduction

Activation of oncogenic proteins is an important mechanism in carcinogenesis. The BRAF gene, located on chromosome 7q34, encodes a serine-threonine kinase that acts downstream of RAS in the RAS/RAF/MEK/ERK signaling pathway involved in regulating cell proliferation and survival. On activation of RAS, the BRAF kinase is activated and sequentially phosphorylates and activates MEK and ERK. A mutation in BRAF leads to constitutive hyperactivation of this pathway through evasion of the inhibitory feedback loop resulting in increased ERK signaling output which drives proliferative and anti-apoptotic signaling (Pratilas et al. 2009). Mutations in BRAF have been reported to occur at high frequency (66%) in melanoma with lower frequencies in colon and other tumours (Davies et al. 2002); BRAF is thus considered to be an important therapeutic target in melanoma (Bollag et al. 2010; Flaherty et al. 2010; Paraiso et al. 2011). Although over 30 single site missense mutations have been identified, 90% occur at nucleotide 1799 resulting in a T-A transition and an amino acid substitution at residue 600 (V600E) in the activation segment (Wan et al. 2004).

In colorectal cancer (CRC) mutations in BRAF have been found in about 9-12% of tumours overall (Di Nicolantonio et al. 2008); (Deng et al. 2004; Jensen et al. 2008). However there is a distinct difference in frequency of BRAF mutations between mismatch repair (MMR) deficient (the microsatellite unstable (MSI-H) tumours) and the mismatch repair intact, microsatellite stable (MSS) tumours (Jensen et al. 2008). This is important clinically as tumours that are MSI-H have a better prognosis (Popat, Hubner, and Houlston 2005). BRAF is mutated in almost all sporadic CRCs with MSI-H (Jensen et al. 2008) but not in tumours arising in patients with an inherited form of MMR deficiency, hereditary nonpolyposis colon cancer (HNPCC), known as Lynch syndrome. Thus a major indication for BRAF mutation testing is for a differential diagnosis of Lynch Syndrome in a CRC that is MSI-H. If BRAF is mutated, the tumour is more likely to be sporadic, rather than the heritable type (Sharma and Gulley 2010).

Mutated BRAF has also been associated with non response to anti-EGFR monoclonal antibody therapy (cetuximab or panitumamab) in metastatic CRC (mCRC) patients (Cappuzzo et al. 2008). In a larger study it was reported that 0/11 patients with a BRAF mutation responded to cetuximab or panitumumab, conversely none of the responders carried BRAF mutations (Di Nicolantonio et al. 2008). BRAF mutation has also been found to be a prognostic factor for poorer outcome in mCRC (Di Nicolantonio et al. 2008); (Price et al. 2011); (Samowitz et al. 2005); (Saridaki et al. 2010); (Souglakos et al. 2009; Tol, Nagtegaal, and Punt 2009); (Van Cutsem et al. 2011).

Although PCR-sequencing to detect BRAF mutations has been the gold standard technique, the improvement in instrumentation for high resolution analysis of PCR amplicon melt curves has opened up the way for the detection of single-base changes in short (approximately 100-200 bp) amplicons (Wittwer et al. 2003). Subsequently an improved method was developed, using melt curve analysis of an oligo-probe, annealing across the region of the mutation (Zhou et al. 2004). As the BRAF mutation is a class IV (T-A) change, we opted for this improved method using commercially available primer and probe sequences. Here we describe the optimisation and validation of this technique for the detection of the BRAF V600E mutation in formalin-fixed paraffin-embedded (FFPE) colorectal tumour tissue and, using the Kaplan-Meier method, the impact of this mutation on survival in the study cohort.

2. Materials and methods

2.1 Tumour collection and processing

Patient samples were obtained from the MAX phase III clinical trial colorectal tumour cohort, described in Price et al. (Price et al. 2011). The MAX study design and eligibility criteria have been reported previously (Tebbutt et al. 2010). Eligible patients were enrolled in this trial between July 2005 and June 2007. After enrollment, patients were randomly assigned to receive capecitabine (C), capecitabine and bevacizumab (CB), and capecitabine, bevacizumab and mitomycin C (CBM). Patient demographic and clinical characteristics are shown in Table 1. Patients in these three groups were evaluated for tumour response or progression every 6 weeks by means of radiologic imaging. Treatment was continued until the disease progressed or until the patient could not tolerate the toxic effects. Samples of tumour tissue from archived FFPE specimens collected at the time of diagnosis were retrieved from storage at participating hospital pathology departments. All patients participating in biomarker studies provided written informed consent at the time of study enrolment. Ethics approval was obtained centrally (Ethics Committee, Cancer Institute of NSW, Australia).

2.2 DNA extraction

DNA was extracted from 1-2 FFPE tissue sections (10 µm) mounted on plain glass slides, with an adjacent section stained with haematoxylin and eosin for reference. In cases that were deemed to have <50% presence of malignant crypts in the section (reviewed by a histopathologist), the tissue was manually dissected to ensure a high proportion of tumour cells. We used a single 10 µm section unless the size of the tissue section was <1 cm, in which case 2 10 µm sections were used. Paraffin was removed by xylene and DNA extracted

using the QIAamp DNA FFPE tissue kit (Qiagen, Valencia, CA, USA), according to the manufacturer's protocol. DNA was quantified using the Nanodrop (Thermo Scientific, Wilmington, DE, USA), ensuring the ratio 260/280 was >1.7.

Baseline characteristic	All patients (%) (n=471)	BRAF MUT (%) (n=33)	BRAF WT (%) (n=280)	P
Age (years)				
Median	67	71	68	0.27
Range	32-86	36-85	32-86	
Sex Male	63	58	64	0.47
ECOG performance status				
0-1	94	88	94	0.11
2	6	12	6	
Capecitabine dosage				
2000mg/m^2/day	67	60	68	0.38
Disease-free interval > 12 months	27	18	30	0.17
Prior adjuvant chemotherapy	22	9	23	0.06
Prior Radiotherapy	13	6	10	0.47
Primary site of cancer				
Caecum	10	21	9	0.02
Ascending colon	10	24	11	0.04
Transverse colon	6	15	5	0.02
Descending colon	3	6	4	0.48
Sigmoid colon	30	18	32	0.11
Recto-sigmoid colon	11	3	13	0.1
Rectum	23	6	22	0.03
Primary tumour resected	79	91	86	0.47
Any metastases resected	10	3	9	0.23
Extent of disease at baseline				
Local disease (colon or rectum)	36	15	33	0.03
Liver metastases	75	62	75	0.19
Lymph node metastases	47	59	45	0.09
Lung metastases	39	21	41	0.03
Bone metastases	4	0	4	0.23
Peritoneal metastases	18	21	16	0.49
Other metastases	10	24	10	0.01

Table 1. Patient demographic and clinical characteristics
(Reproduced with permission from the Journal of Clinical Oncology).

2.3 Mutation analyses

Mutation status of BRAF was determined using high resolution melting analysis (HRM) PCR on the Rotorgene 6000 real-time instrument (Qiagen). BRAF HRM PCR (119 bp amplicon) was performed on 10 ng DNA in triplicate reactions using SsoFast™ EvaGreen® Supermix (Bio-Rad Laboratories Inc., Hercules, USA) and a primer/probe combination (RaZor® probe HRM assay, PrimerDesign, Southampton, UK). The sequences were 5'ATGAAGACCTCACAGTAAAAATAGG (sense), CTCAATTCTTACCATCCACAAAATG (antisense) and 5'GTGAAATCTGGATGGAGTGGGTCCCATCA (probe). Appropriate mutant and wild type (WT) controls were included. A 'touch-down' PCR cycling protocol was used for the first 9 cycles to avoid primer mis-priming events and, due to the asymmetric design, 50 cycles were performed according to the manufacturer's protocol. The sensitivity of detection of mutant sequences was determined by assaying dilutions (100%, 50%, 25%, 12.5%, 6.25%) of a tumour DNA sample, with known homozygous BRAF mutation status, in BRAF WT cell line DNA. Using the Rotor Gene 6000 (Qiagen) software analysis features for HRM, patient samples (n=315) were classified as having mutated (MUT) or WT BRAF respectively. Direct PCR sequencing was used to validate all mutant BRAF results and an additional 106 randomly chosen samples (45% of samples in total). The primers for BRAF sequencing reactions were designed in-house and obtained commercially (Geneworks, Thebarton, SA, Australia): 5'AATGCTTGCTCTGATAGGAAAA (sense) and 5'AGTAACTCAGCAGCATCTCAGG (antisense). PCR products were purified using ExoSAP-IT (GE Healthcare, Buckinghamshire, UK) to remove unwanted deoxynucleotides and primers according to the manufacturer's protocol. Sequencing was performed by Flinders Sequencing Facility (Flinders Medical Centre, Bedford Park, SA, Australia) using BigDye Terminator v3.1 chemistry and the Applied Biosystems 3130xl Genetic Analyser (Life Technologies, Carlsbad, CA, USA).

2.4 Statistical analyses

All randomly assigned patients for whom data on BRAF mutation status were available were included in the analysis (n=313). PFS, the primary endpoint, was defined as the time from randomisation until documented evidence of disease progression, the occurrence of new disease or death from any cause. The secondary endpoint was overall survival (OS), defined as the time from randomisation until death from any cause. The PFS and OS of patients according to BRAF status were summarised with the use of Kaplan–Meier curves, and the difference between these groups was compared with the use of the log-rank test. All reported P values were two-sided.

3. Results and discussion

Although significantly less DNA was isolated from the microdissected sections (P=0.0001), the range of values obtained overall, 60 ng -31.3 µg, meant that all samples were well within the amount required for the PCR (30 ng) (Figure 1).

In interpreting the HRM results, the first criterion of robust PCR amplification must be met (Figure 2A), so that the duplicates must show close Ct values (standard deviation <0.5) otherwise samples must be excluded from the HRM analysis and the PCR repeated.

Samples that show poor amplification with late Ct values may give erroneous results on HRM as shown in Figure 2B. The samples in the boxed area need to be excluded from the analysis to avoid misinterpretation of the difference plot as mutant calls. The poor amplification of a DNA sample may be due to the presence of inhibitors, and we have found that subsequent isolation of DNA from microdissected sections gave much better, more reproducible amplification results. This also suggests that minimising the amount of paraffin in the DNA preparation may be contributing to the improvement in PCR performance.

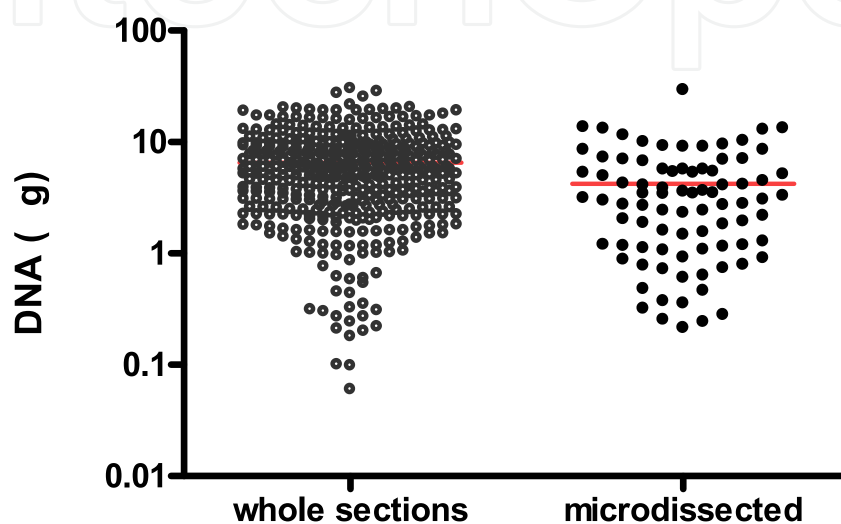


Fig. 1. Dot plot of DNA yields. The average amount of DNA obtained from whole sections was 6.5±0.25 μg and from manually microdissected sections 4.2±0.48 μg.

The positioning of the normalisation regions 1 and 2 in the first HRM analysis window is also a very important parameter in the correct calling of genotypes. This is user-defined and performed separately for HRM analysis of the probe region or the amplicon region. The correct positioning may be determined by monitoring the normalised graph to show the best separation of mutant versus WT curves.

To determine the level of sensitivity of detection, serial doubling dilutions of a tumour sample carrying a homozygous BRAF V600E mutation were tested. The difference graph, normalised to the WT control, shows that the mutation could be detected down to a dilution of 6.25% mutant DNA in WT DNA (Figure 3A). Although there is a distinct difference between the WT control used for normalisation and the 6.25% and 12.5% dilutions, in practice the software cannot call these with any confidence. From the normalised graph and the melt curves graph (Figure 3B and 3C), 25% mutant DNA appears to be the lower limit of detection. However to increase the probability of correctly assigning a genotype we aimed for at least 50% epithelial tumour cells, hence all of the tumour tissue in the cohort was reviewed to ensure at least 50% epithelial tumour cells were present. Manual microdissection was performed in 1/5 of the cohort to ensure >50% enrichment of tumour cells, relative to muscularis mucosa and other cell types such as lymphoid aggregates, in the sample.

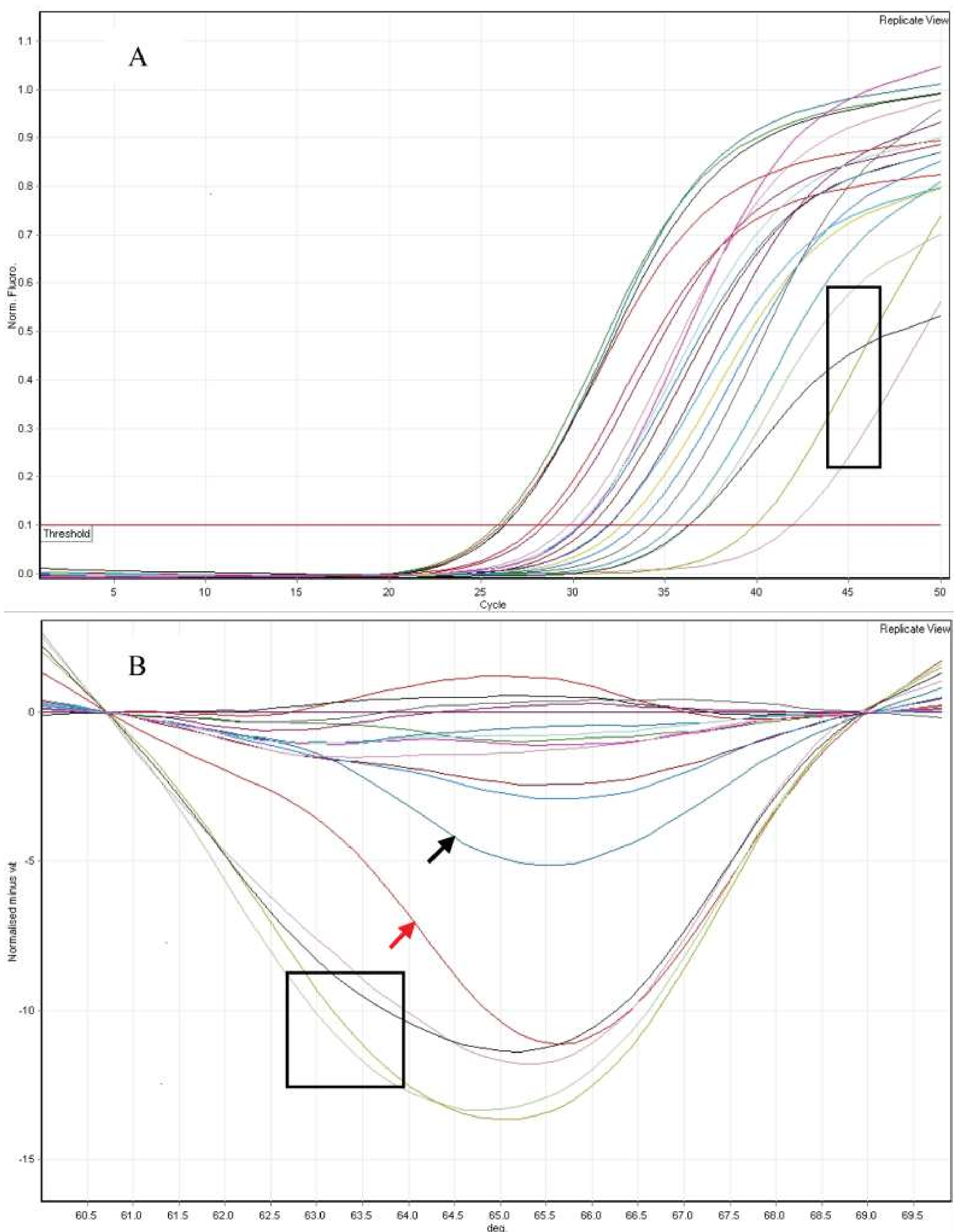
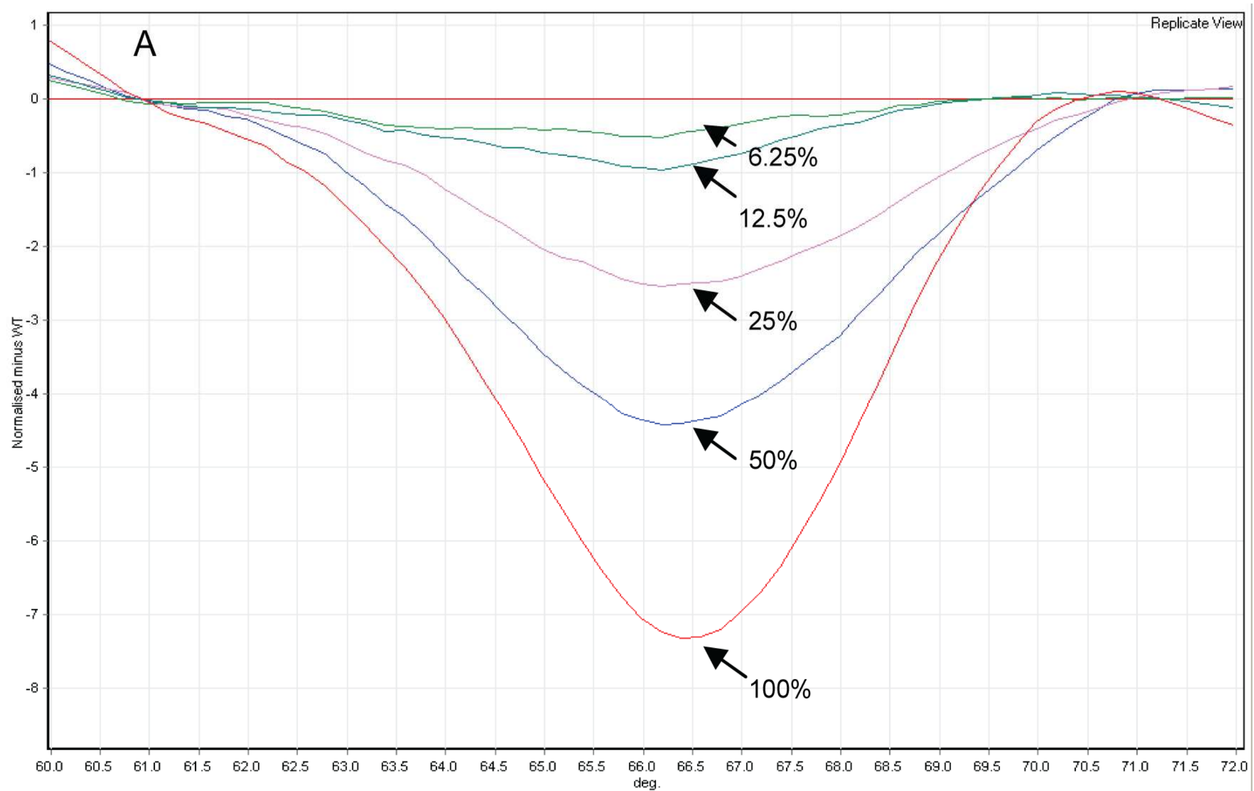


Fig. 2. A: amplification curves; B: difference plot normalised to WT. The boxed curves in A show samples with aberrant late amplification. The same samples boxed in B show the abnormal difference plots that could be incorrectly interpreted as mutant. Black arrow in B points to the heterozygous mutant control, red arrow shows the homozygous mutant control.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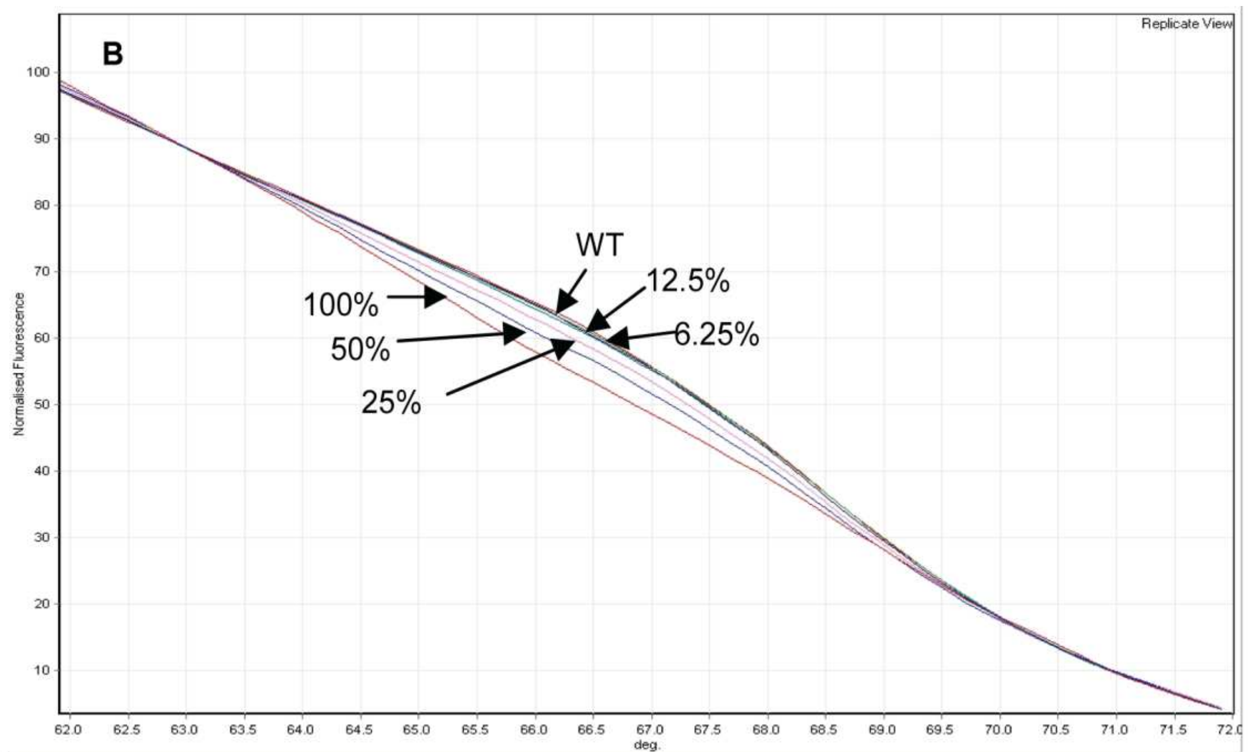
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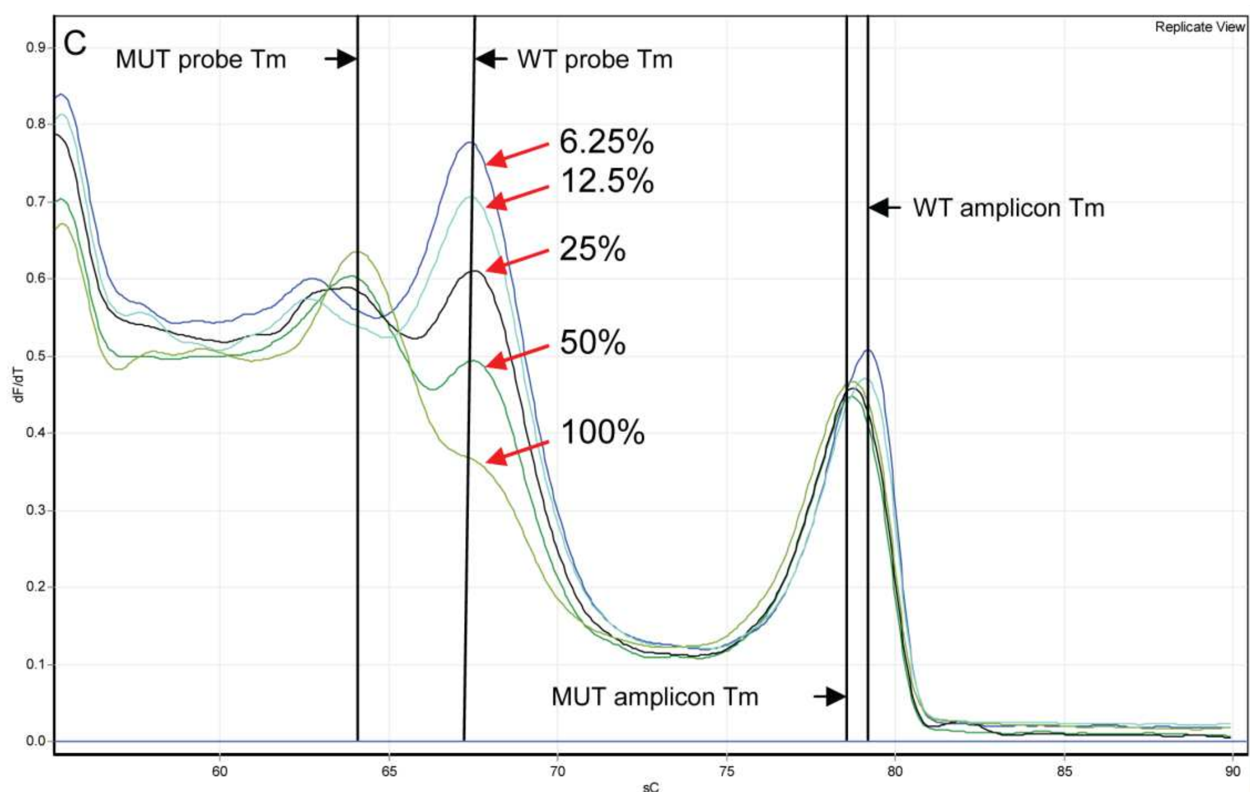

Fig. 3. A: Difference plot normalised to WT, with dilutions of homozygous MUT control DNA in WT DNA shown in replicate view (average of 3 for each dilution). Arrows indicate the plots for the dilutions of MUT control DNA in WT DNA from 100% MUT to 6.25%MUT; B: Normalised melting curves of the probe region. From this view it was not possible to distinguish the 12.5% or 6.25% dilutions of mutant sequence from WT; C: Melt curve showing Tm's for both the probe region and amplicon. The probe region HRM analysis was much easier to interpret than the amplicon HRM, however the 12.5% and 6.25%dilutions were indistinguishable from WT pattern.

We have found that it was of critical importance to select the control genotypes (WT or mutant) for the normalisation carefully. The DNA of these controls needed to be extracted from a similar tissue (i.e. colonic tissue FFPE), and be processed in exactly the same way as the test samples. Using cell line derived DNA as the controls resulted in too many mutation calls with low confidence (false positives), however when we used tumour samples of known *BRAF* status as the controls, the confidence of the software calls of the test samples reached >99%. Often we found it was more informative to look at the shape of the curves in the difference plot, even if a curve deflected away from the horizontal normalised line, the angle of deflection was much greater for mutant genotypes and shallower for WT (Figure 4). This visual interpretation usually correlated with the software calls and was a useful adjunct in interpretation where the confidence of the software calls was low.

Sequencing was used to validate the results and correlated with the HRM results. In some cases though sequencing showed a very small A peak which could be overlooked whereas HRM showed a very convincing shift and was called as a mutation with 99% confidence. An example is shown in Figure 5.

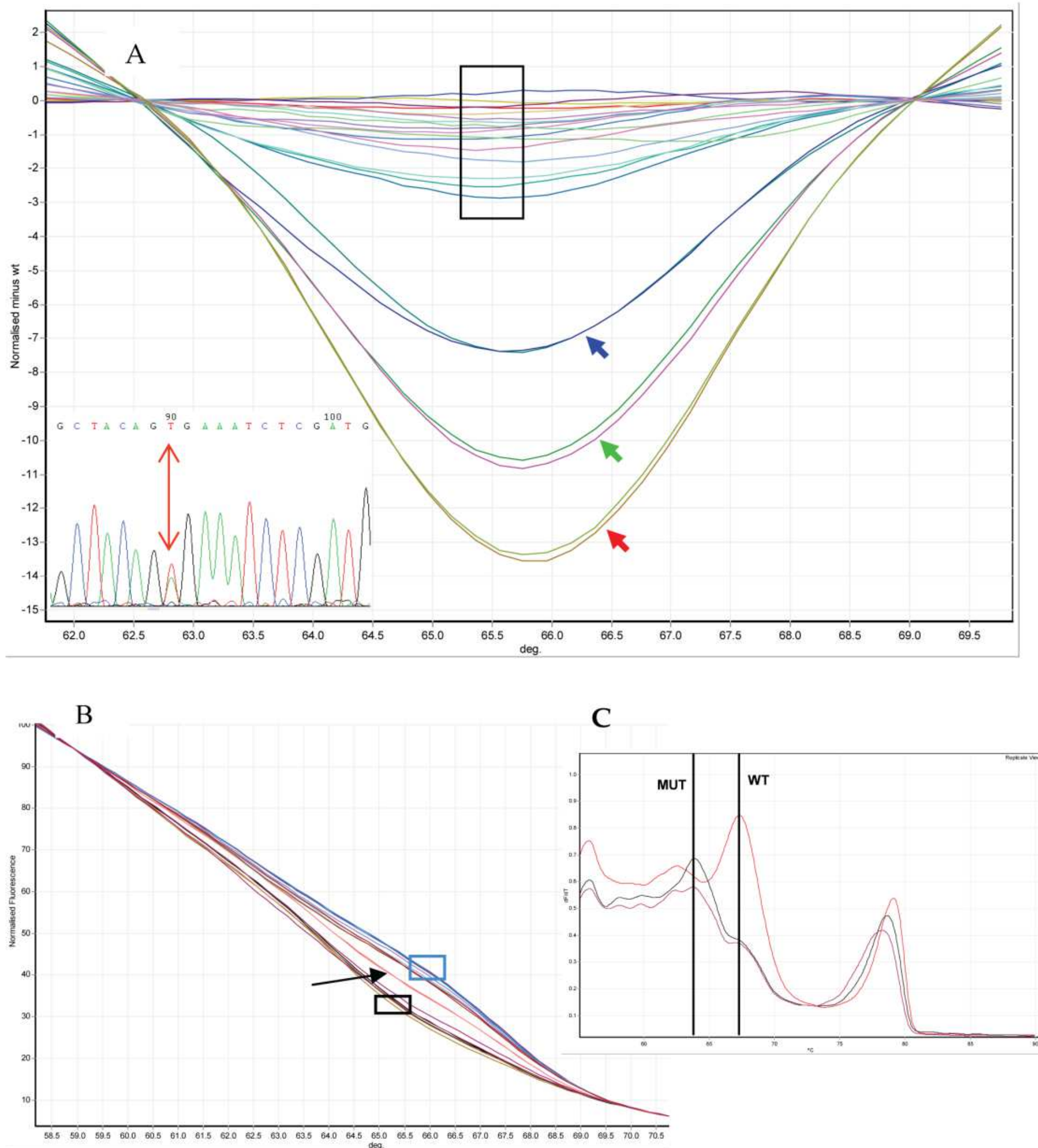

Fig. 4. Sequencing result and corresponding HRM analysis. A: Difference graph normalized to WT control (duplicates), blue arrow shows heterozygous control, green arrow pt 109, red arrow homozygous MUT control. Boxed area shows WT samples. Inset is the sequencing trace (Chromas Lite software) of patient (pt) 109, red arrow showing mutant (A) amongst WT (T) sequence. B: Normalised melt curve of probe region; black boxed area shows homozygous mutant control and 2 samples including pt 109, arrow points to the heterozygote mutant control, blue box shows WT control and WT samples. C: Melt curve analysis, red trace WT, black trace homozygous MUT control, purple trace pt 109.

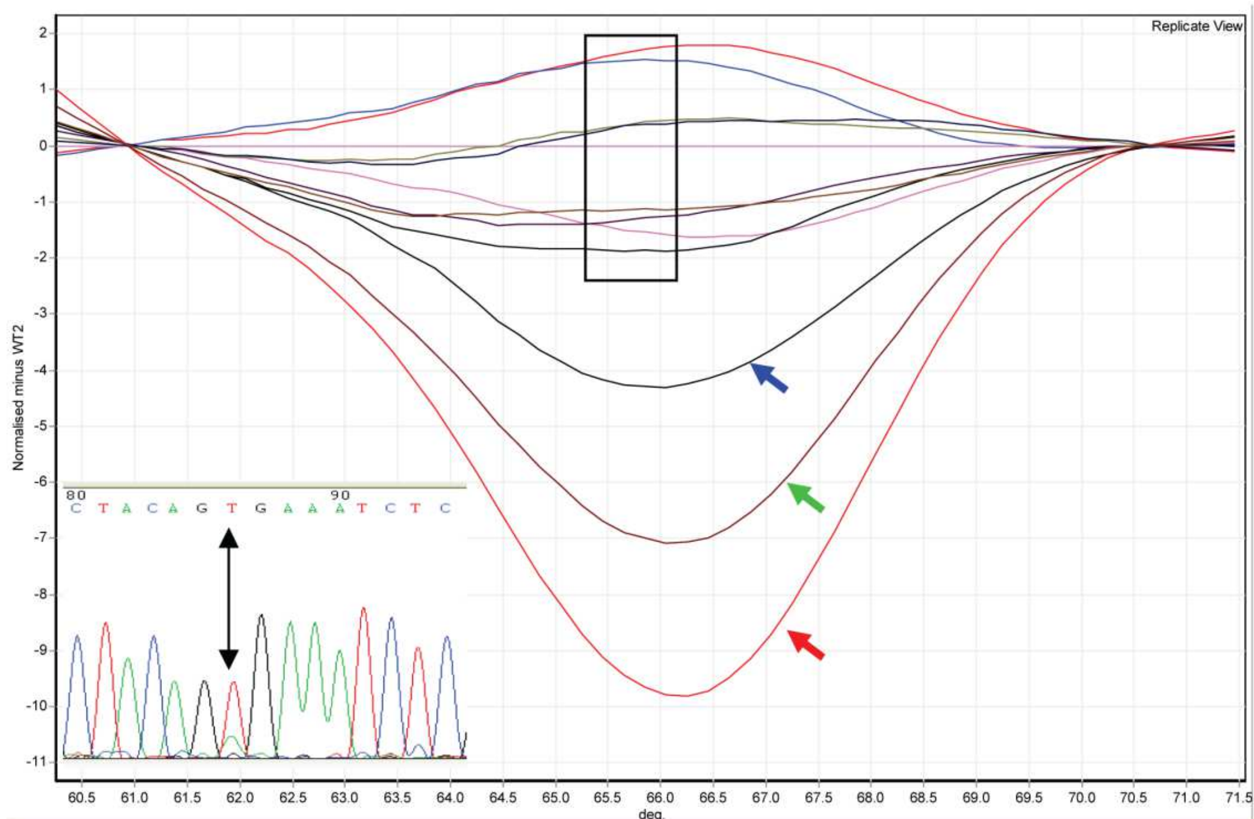

Fig. 5. An example of a sequencing result (pt 269) called WT (T) by the sequencing software that did in fact show a small A peak. The difference plot of the HRM analysis (normalized to WT control) showed a definite downward shifted curve (green arrow) between the homozygous BRAF MUT control (red arrow) and the heterozygous control (blue arrow). The boxed curves show the WT samples.

Of 471 patients who underwent random assignment, a total of 315 tumour specimens (n=103 from the capecitabine group, n=111 from the CB group, and n=101 from the CBM group, accounting for 66.9% of the total study population) were examined for *BRAF* mutation status by HRM. *BRAF* V600E mutations were detected in 10.5% of 313 tumours (2 samples were not evaluable). A proportion of samples were also genotyped using sequencing and showed 100% correlation with the HRM result.

A total of 313 patients were included in the survival analysis with a median follow-up time of 26.5 months (range, 0.4 to 37.6 months). There was no significant difference in PFS between patients with WT tumours and those with mutated tumours. The median PFS was 4.5 months among the patients with V600E tumours as compared with 8.2 months among those with WT tumours (HR: BRAF WT *vs* MUT, 0.80; 95% CI, 0.54 to 1.18; P=0.26). In contrast, there was a significant difference in OS between patients with WT tumours and those with V600E tumours. The median OS was 8.6 months among the patients with mutated BRAF tumours as compared with 20.8 months among those with WT tumours (HR: BRAF WT *vs* MUT, 0.49; 95% CI, 0.33 to 0.73; P=0.001) (Figure 6). BRAF status remained prognostically significant after adjustment of pre-defined baseline prognostic factors

including age, sex, ECOG performance status, inoperable local disease, and prior chemotherapy (HR: BRAF WT *vs* MUT, 0.45; 95% CI, 0.30 to 0.68; P<0.0001).

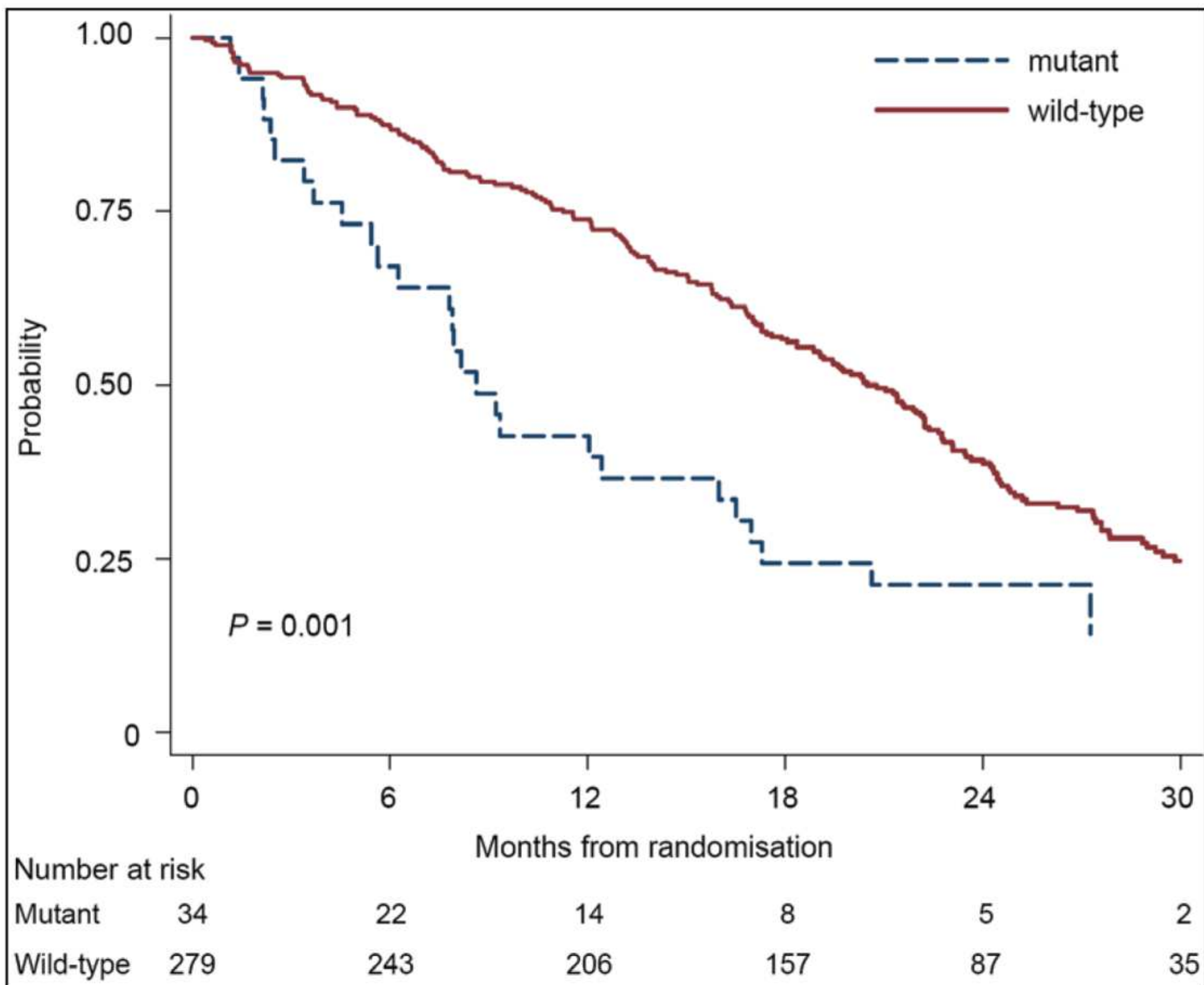

Fig. 6. Kaplan-Meier analysis for overall survival comparing patients WT or MUT for *BRAF* . The curves are significantly different (P=0.001, log-rank test). Reproduced with permission from the Journal of Clinical Oncology.

4. Conclusion

HRM analysis is a useful fast technique to determine BRAF mutations using the platform of real-time PCR. It is both reproducible and reliable provided the preceding guidelines are followed and rigorous attention is given to the PCR performance as well as to the use of the software analysis package. Here we have described how the technique can be applied to the analysis of DNA extracted from archived FFPE tissue sections, which in many cases is the only source of tumour tissue available for retrospective analyses. The survival analysis showed that metastatic CRC patients with tumours carrying the V600E mutation had significantly poorer overall survival outcomes compared to those without the mutation. This HRM analysis could equally be applied to the assessment of tumours from patients diagnosed with other diseases known to have a significant BRAF mutation rate.

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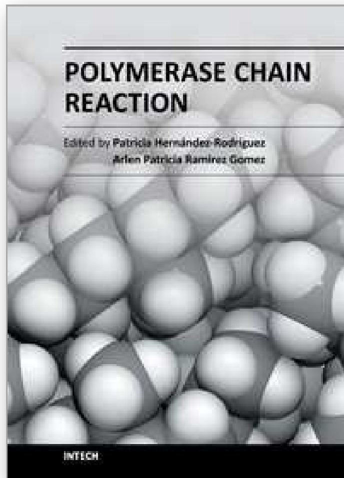

Polymerase Chain Reaction

Edited by Dr Patricia Hernandez-Rodriguez

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This book is intended to present current concepts in molecular biology with the emphasis on the application to animal, plant and human pathology, in various aspects such as etiology, diagnosis, prognosis, treatment and prevention of diseases as well as the use of these methodologies in understanding the pathophysiology of various diseases that affect living beings.

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InTech Europe
University Campus STeP Ri
Slavka Krautzeka 83/A
51000 Rijeka, Croatia
Phone: +385 (51) 770 447
Fax: +385 (51) 686 166
www.intechopen.com

InTech China
Unit 405, Office Block, Hotel Equatorial Shanghai
No.65, Yan An Road (West), Shanghai, 200040, China
中国上海市延安西路65号上海国际贵都大饭店办公楼405单元
Phone: +86-21-62489820
Fax: +86-21-62489821

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