Do somatic USP8, USP48 and BRAF mutations differ in their genotype-phenotype correlation in Asian Indian patients with Cushing's disease?

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Abstract

Purpose

To estimate the prevalence of *USP8*, *USP48* and *BRAF* mutations in patients with Cushing's disease (CD) from the Indian subcontinent, and determine their genotype-phenotype correlation.

Methods

We prospectively recruited 46 patients with CD who underwent surgery between September 2015 and July 2019 at our institute. Fresh frozen tumour tissue was obtained in all patients. Using Sanger sequencing, the presence of somatic *USP8* mutations was documented and the frequency of *USP48* and *BRAF* mutations in *USP8* wild-type corticotroph adenomas was determined. Clinical, hormonal and surgical data were then compared between *USP8*-, *USP48*- and *BRAF*-variant carriers and patients with wild-type tumours.

Results

Signature *USP8* mutations were detected in 17 (37%) patients. Of the 29 *USP8* wild-type adenomas, 4 (13.8%) harboured *USP48* mutations, one of them being a splice-site mutation that has previously not been described. *BRAF* mutations were not found in any of the 29 patients. Corticotroph adenomas with *USP8* mutations had a higher incidence of Crooke's hyaline change than wild-type tumours (70.6% vs. 37.9%, $p = 0.032$). Adenomas with *USP48* mutations had a higher rate of cavernous sinus invasion than their wild-type counterparts (50% vs. 4%, $p = 0.042$). No other significant phenotypic difference could be established between mutant and wild-type tumours.

Conclusions

The prevalence of *USP8* mutations in our series of patients with CD was 37%. The prevalence of *USP48* mutations in *USP8* wild-type adenomas was 13.8%, including a novel splice-site mutation. *BRAF* mutations were not found in any *USP8* wild-type tumour. *USP8*-mutants showed significantly more Crooke's hyaline change and *USP48*-mutants were more likely to demonstrate cavernous sinus invasion.

Introduction

Cushing's disease (CD) is a type of adrenocorticotrophic hormone (ACTH) dependant Cushing's syndrome (CS) caused by hypersecretion of ACTH by pituitary adenomas. Extensive research has been done to explore the possible molecular mechanisms responsible for it but the exact aetiopathogenesis remains unclear.

Several recent publications have identified somatic mutations in the ubiquitin-specific protease 8*(USP8)* genes of 21–62% patients with CD.[1–5] These mutations in exon 14 of the *USP8* gene, via increased deubiquitinase (DUB) activity, have been shown to increase the expression of epithelial growth factor receptors (EGFR), thereby up-regulating EGFR-induced proopiomelanocortin (POMC) transcription and ACTH secretion. While the link between EGFR signalling and *USP8* mutations was established in in-vitro studies,[1, 3] other authors failed to demonstrate increased EGFR expression on immunohistochemistry (IHC) in *USP8* mutated corticotroph adenomas.[6] A recent publication[7] addresses this by demonstrating that mutant adenomas also suffer dysregulation of a number of other proteins namely p27/kip1, CABLES1, HSP90, and activated CREB; all of which have been previously found to be associated with either increased ACTH production or increased cell proliferation.[8]

Further research revealed that in *USP8* wild-type corticotroph adenomas, mutations in *USP48* (10–23%) as well as *BRAF* (1–16%), which are also known to increase POMC transcription, might be the drivers of ACTH over-production.[9, 10]

What is evident from the literature is that there is a wide variation in the reported prevalence of *USP8*, *USP48* and *BRAF* mutations from different countries. There is also limited clinical, hormonal and radiological data available on patients harbouring these mutations. Few studies have shown that *USP8*-mutant corticotroph adenomas occur more frequently in women, are smaller in size, are less invasive, and are more likely to lead to disease remission following surgical resection.[2, 5, 6] A single study showed that *USP48*-variant adenomas were smaller in size.[10]

In this study we aimed to determine the prevalence of signature and novel mutations in these genes in a cohort of South Asian patients for the first time, and also look for definite genotype-phenotype correlation in terms of their clinical, hormonal and radiological profile.

Materials And Methods

Design and setting

This was a prospective study conducted at the Christian Medical College, Vellore with the collaborative efforts of the Departments of Neurosurgery, Endocrinology and Pathology. It was approved by the Research and Ethics Committee of the Institutional Review Board (IRB Min No: 9628, dated 01.09.2015). The design was cross-sectional, to estimate the frequency of somatic *USP8* mutations in patients with CD and frequency of *USP48* and *BRAF* mutations in patients with *USP8* wild-type tumours.

Clinical data

Between September 2015 and July 2019, all patients diagnosed to have CD using standard clinical and biochemical criteria and who had discernible tumours on dedicated magnetic resonance imaging (MRI) sequences of the pituitary gland were enrolled in the study after obtaining informed consent in the case of adults, and assent in the case of minors. All patients underwent endoscopic transsphenoidal resection

of their corticotroph adenomas. If patients were on ketoconazole, the drug was stopped 2 weeks prior to surgery. Using the surgical strategy previously published by us,[11] either selective adenomectomy, enlarged adenomectomy, hemihypophysectomy or subtotal tumour resection was done, depending on whether tumour margins were clearly defined intraoperatively. Perioperative steroids were not administered in order to enable early evaluation of remission. Postoperatively, patients were started on steroids only if their 8 AM cortisol level dropped < 5 µg/dl or if they demonstrated symptoms of hypocortisolaemia or hyponatraemia. Patients were asked to review 3 months following surgery and thereafter every year. Remission was defined by either the presence of a basal cortisol of < 5 µg/dl with requirement for steroid replacement therapy, or a basal cortisol > 5 µg/dl but with suppression to < 1.8 µg/dl with the 1 mg overnight dexamethasone suppression test

Pathological evaluation and mutational analysis

Each biopsy specimen obtained for a patient was equally divided for histopathological examination and genetic sequencing. Tissue for histopathological examination was fixed in 10% buffered formalin and embedded in paraffin. Standard haematoxylin-eosin and reticulin staining was employed. Disruption of the normal acinar architecture along with the presence of monomorphic cells was considered as confirmation of tumour. If tumour could not be identified on initial sections, serial sectioning and examination with both haematoxylin-eosin and reticulin staining was done. Periodic acid Schiff (PAS) staining and IHC for ACTH (1:150, Biogenex, San Ramon, CA), other anterior pituitary hormones, and MIB-1 labelling index were performed on all surgical specimens using 5-µm sections. PAS and cytokeratin immunostainings were also used to confirm Crooke's hyaline change in normal adenohypophysis.

DNA was extracted from the fresh frozen samples of tissue that was confirmed to be tumorous on histopathology. The samples, which were stored at -70˚C, were first thawed and a standardised protocol for DNA extraction from tissues was followed. The QIAamp DNA Mini Kit (Qiagen) was used for all extractions. The DNA was quantitated using a NanoDrop™ microvolume spectrophotometer (NanoDrop Technologies) and the 260/280 ratio was determined. Samples with a poor ratio or quantity were amplified with primers targeting a 133 bp region of an endogenous control, before they were subjected to further analysis.

The polymerase chain reaction (PCR) for the two primer sets of exon 14 (*USP8*) were performed using previous published primers.[2] For *USP48* and *BRAF*, the primer sequences used were based on that employed by Chen *et al.*[9] and Ferchichi *et al.* respectively.[12] Details of primer sequences and thermal cycling conditions are elaborated in Supplementary Table 1. All reactions were carried out in 25 µl volume. The following thermal cycling profile was employed for all PCRs: 95^0C for 8 minutes, 95^0C for 30 seconds, optimized anneal for 30 seconds, 72^0C for 1 minute and final extension of 72^0C for 10 minutes. The PCR product was detected using a 1.5% agarose gel. Sanger sequencing of both the sense and antisense strands of products for both primer sets were performed with an automated DNA sequencer (ABI PRISM 310 genetic analyser) using the ABI PRISM BigDye Terminator Cycle Sequencing Ready

Reaction Kit (Applied Biosystems). Mutational analysis was performed by comparing the sequence with the wild type and by looking for the presence of all known mutations in this exon.

Data collection and statistical analysis

A comprehensive proforma was used to record the clinical, biochemical and radiological parameters of each patient. Details of the surgery, histopathological examination, mutational analysis and postoperative complications were also documented. Follow-up data was regularly updated as and when patients reviewed in the outpatient clinic. Mean with standard deviation (SD) / median with inter-quartile range (IQR) were used for continuous variables as applicable. Frequency and percentage were used for categorical variables. Prevalence was expressed as a proportion with 95% CI. Categorical variables were compared using Chi-square testing. Continuous variables were compared using Student's t-test if normal assumptions were satisfied; otherwise the Mann-Whitney U test was used. Multivariate penalised logistic regression was done on variables which had p-values < 0.2 on bivariate analysis. Statistical analysis was performed using IBM SPSS Statistics for Windows, version 21 (IBM Corp., Armonk, N.Y., USA) and Stata version 15 (StataCorp LLC, TX, USA).

Results

During the study period, a total of 49 patients were recruited for the study, however 3 patients were subsequently excluded because adequate fresh frozen tumour tissue was not available for DNA extraction.

Preoperative data

The mean age of our study population was 29.5 ± 8.2 (range 12-48) years and included 3 adolescents aged 12, 13 and 15 years. Thirty eight (82.6%) were females. Six (13%) patients had been operated elsewhere earlier and presented to us with recurrent/persistent CD. The mean duration of symptoms was 2.6 (range 0.2-10) years. Nineteen (41.3%) patients were on preoperative ketoconazole for a mean duration of 2.5 (range 0.3-14) months. Control of hypercortisolaemia with ketoconazole was achieved in only 2 out of these 19 patients.

There were 29 (63%) microadenomas and 17 (37%) macroadenomas. Of the macroadenomas, 13 were Wilson-Hardy Grade A tumours, 3 were Grade B and 1 was grade E. Four (8.7%) adenomas showed cavernous sinus invasion. Table 1 summarises the preoperative data of all patients.

Surgical details

Gross total resection could be achieved in 44 (95.7%) patients. Only subtotal resection was achieved in 2 (4.3%) patients because of cavernous sinus invasion and bleeding that limited further resection. Of the patients who had gross total resection, 17 (37%) underwent selective adenomectomy, 25 (54.3%) underwent enlarged adenomectomy and 2 (4.3%) underwent hemihypophysectomy.

Remission and recurrence rates

At 3 months follow-up, 37 (80.4%) out of 46 patients were in remission. Only 4 out of 8 (50%) males entered remission, while 33 (86.8%) females achieved the same. Amongst those who went into remission, long term follow-up was available in 31 patients with a mean duration of 25.3 ± 13.6 (range 6-50) months. Seven (22.6 %) of these 31 patients (6 female and 1 male) subsequently had disease recurrence with a mean time to recurrence of 20.1 ± 7.4 (range 8-28) months.

Mutational analysis

In all 46 patients, the diagnosis of corticotroph adenoma was confirmed by histopathological examination and immunopositivity for ACTH. On DNA sequencing, 17 (37%) patients had somatic mutations of the *USP8* gene in the mutational hotspot of exon 14 at the 14-3-3 binding motif. Of these, 11 were missense mutations while the remaining were frameshift mutations. All mutations were located in the amino acids (AA) 718-720 with the most common being p.Pro720Arg and p.Ser719del fs. The full list of mutations is found in Supplementary Table 2. Figure 1(a) shows the DNA sequencing chromatogram of a *USP8* wild-type tumour, Figure 1(b) shows the chromatogram of a *USP8*-mutant with a p.Pro720Arg mutation, and Figure 1(c) shows the chromatogram of frameshift mutation (p.Ser719del fs).

In the 29 *USP8* wild-type adenomas, *USP48* mutations were present in 4 (13.8%), with three missense mutations (p.Met415Ile) and1 splice-site mutation (p.Pro433IVS+2 T>A). There were no *BRAF* mutations in any of our patients. Figure 2(a) depicts the DNA sequencing chromatogram of patient with no *USP48* mutation, Figure 2(b) is the chromatogram of a patient with a p.Met415Ile mutation in exon 10 of the *USP48* gene and Figure 2(c) shows a *USP48* splice-site mutation (p.Pro433IVS+2 T>A).

Genotype-phenotype correlation of patients with USP8 and USP48 mutations

Tables 2 and 3 compare the clinical, radiological, biochemical and surgical characteristics of the *USP8*-mutants and *USP8* wild-type tumours. The only significant difference between both groups was an increased presence of Crooke's hyaline change in the adjoining hypophysis of the mutated tumours (70.6% vs. 37.9%, p=0.032). There was larger number of macroadenomas in the *USP8*-mutant group but this fell just short of statistical significance (52.9% vs. 27.6%, p=0.085). Only 1 out of 8 (12.5%) males harboured the mutation while 16 out of 38 (42.1%) females had mutated tumours although this was not statistically significant. Also not statistically significant but important to note, was a higher rate of disease recurrence in patients with *USP8*-mutant tumours (33.3% vs. 18.2%, p=0.38). On multivariate analysis, Crooke's hyaline change remained significantly higher in patients with mutated tumours (OR 5.05, 95% CI 1.02 – 25; p=0.047). All other variables were similar in both groups.

The comparison between patients with *USP48*-mutants and *USP48* wild-type tumours is made using bivariate analysis in Tables 4 and 5. The only significant difference between both groups was a higher rate of cavernous sinus invasion in the *USP48*-mutants (50% vs. 4%, p=0.042). Tumours with *USP48* mutations recurred more frequently than their wild-type counterparts (33.3% vs. 15.8%; p=0.470),

although this was also not statistically significant like in the case with the *USP8*-mutants. On multivariate analysis, there was no variable that was significantly different between the 2 groups.

Discussion

Prevalence of USP8, USP48 and BRAF mutations

The prevalence of *USP8* mutations in CD reported thus far ranges from 21-62% (Supplementary Table 3).[1–6,13,14] Our study, the first from South Asia, found a prevalence of 37%. The *USP8* mutations found in our patients have been previously described and no novel mutations were discovered, however, we found a hitherto unreported *USP48* splice-site mutation - p.Pro433IVS+2 T>A.

Somatic mutations of both *USP48* and *BRAF* genes in patients with CD were reported only in 2018.[9,10] The pooled incidence of *USP48* and *BRAF* mutations in these two studies was 41/308 (13.3%) and 16/227 (7%).[15] In our study, the prevalence of *USP48* mutations in *USP8* wild-type adenomas was 13.8%, almost identical to that in the literature. Of interest is that almost all *USP48*- and *BRAF*-variants were noted only in patients with *USP8* wild-type tumours, although they themselves were not mutually exclusive.

Another important finding in our study was that not a single *BRAF* mutation was detected in the 29 patients with *USP8* wild-type status. The absence of BRAF mutations in our patients with CD is in stark contrast to the 16.5% prevalence reported from China[9] but is similar to 1% prevalence amongst Caucasians.[10] Ethnicity may also have a bearing on the presence of *USP8*-variants,[1,2,5,7,10,14] as is evident from the higher prevalence of *USP8* mutations (62%) in the Chinese population[3] as compared to that in the European population (21-48%).

Genotype-phenotype correlation of USP8-variants

Several studies have shown a significantly higher, if not exclusive occurrence of *USP8*-variants in women.[2,3,6,10,13,16] This was however not the case in the series by Albani *et al*.,[14] who found that there was no significant gender association (*USP8* mutations in 39% of females vs. 30% of males). In our series, only 1 male out of 8 (12.5%) had a *USP8* mutation while 16/38 (42.1%) females carried *USP8*-variants. No male patient out of the 4 with *USP8* wild-type CD harboured *USP48* mutations. While this difference did not attain statistical significance due to the relatively small sample size, there seems to be a clear tendency towards female preponderance and might explain, at least partially, the well-known female predilection in CD which so far has not been adequately understood. Perez-Rivas *et al*.[2] first proposed that oestrogens could demonstrate a growth-stimulating effect on *USP8*-mutated corticotrophs, a hypothesis supported by the fact while a balanced sex-ratio exists amongst paediatric patients with CD[17] in adults there is a distinct female predominance of the disease.[11] In addition, the demonstration of oestrogen receptors on corticotroph cells along with the stimulatory effect of oestradiol on murine corticotroph cell proliferation via EGFR signalling might be a reason for a higher incidence of CD in females.[18,19]

Some reports indicate that *USP8*-mutated tumours occur in younger adults [2,7,14] and older children,[13] however, we and others[3–6] found no significant age difference between carriers of *USP8*-mutant and wild-type adenomas. BMI was found to be significantly higher in patients harbouring *USP8*-mutant tumours in two studies,[2,10] while another found that BMI was lower amongst patients with *USP8*-mutant tumours.[13] We could not demonstrate any statistically significant difference in BMI between the 2 groups.

We found that the *USP8*-mutants in our series tended to be larger than their wild-type counterparts, although this did not achieve statistical significance (p=0.09). Findings on the issue of tumour size vary considerably, with variant-carrying tumours being reportedly smaller and less invasive in some studies, [3,6] while others have observed a greater size in *USP8*-mutant microadenomas compared with wild-type microadenomas.[2,5] This difference may be at least partially attributed to a selection bias in some studies. For example, Hayashi and colleagues[6] oversampled Crooke's cell adenomas, while the Chinese series[3] included a relatively large proportion of invasive tumours (>20%) and giant adenomas. Also worth noting is that none of the series reported so far mirror the ratio between micro- and macroadenomas normally found in CD, i.e. less than 10-20% macroadenomas, probably a consequence of the need for adequate pathological specimens to perform DNA or RNA sequencing.

With regards to the biochemical characteristics of these patients, one study[2] found that patients with *USP8* mutations demonstrate increased suppression of cortisol after 8 mg dexamethasone while another found significantly lower plasma ACTH levels in these patients.[6] Ma *et al*.[3] found that ACTH secretion was higher in patients carrying *USP8* mutations. We failed to establish any significant difference between wild-type and mutated tumours in the biochemical parameters studied, a finding corroborated by others. [2,5,13,14]

However, despite revealing no significant difference between the ACTH, UFC and serum cortisol in *USP8*-variant and *USP8* wild-type we did find that the proportion of patients with Crooke's hyaline change in the adjoining adenohypophysis was significantly higher in the *USP8*-variant group. Crooke's hyaline change is typically seen with higher levels of hypercortisolism,[20] so it may be conjectured that patients with *USP8*-mutant adenomas may have experienced severe hypercortisolism at some point of their illness.

The remission rates of CD was similar in the *USP8*-variant and wild type tumors as reported by others. [2,3,14] Some authors[5,6] note a higher remission rate in patients with *USP8* mutations while others[13,14] report higher recurrence rates in mutated tumours. Another study found parallel recurrence rates, but a shorter mean time to recurrence in the *USP8*-variant adenomas.[3] We too found a higher recurrence rate amongst our patients with a *USP8* mutation (33% vs. 18%), although it was not statistically significant due to the small sample size.

Genotype-phenotype correlation of USP48-variants

The study by Chen *et al*.[9] could not demonstrate any significant clinical, biochemical or radiological differences between patients with *USP48*-variants and *USP48* wild-type corticotroph adenomas. However

a subsequent study reported that *USP48*-mutant adenomas were smaller than wild-type adenomas.[10] In our series, the occurrence of cavernous sinus invasion was significantly higher in the 4 patients with *USP48*-mutant adenomas (50% vs. 4%, p=0.042). This difference has not been reported by any other study, however further research is required on these less common mutations before any meaningful conclusions can be drawn. The literature available on the genotype-phenotype correlation of CD patients with *USP8* and *USP48* mutations is summarised in Table 6.

Strengths and limitations of this study

This single-centre, prospective study from the Indian subcontinent, determines the prevalence of *USP8*, *USP48* and *BRAF* mutations in patients with CD using fresh frozen tumour tissue that provided uniformity in the quality of tissue used for mutational analysis. The main limitation of the study was its relatively small sample size that precluded the establishment of statistically significant phenotypic differences between patients with *USP8*-/ *USP48*-variant tumours and their wild-type counterparts. Moreover, only mutations in the previously described mutational hotspots of the *USP8, USP48* and *BRAF* genes were tested for. Next-generation sequencing (NGS) may have identified additional mutations in our series, as was seen in the study by Ballmann and colleagues,[4] where NGS detected a few *USP8* mutations that were missed on Sanger sequencing.

Conclusions

The prevalence of somatic *USP8* mutations in corticotroph adenomas was 37%. The frequency of *USP48* mutations in patients with *USP8* wild-type adenomas was 13.8% while *BRAF* mutations were not found in any of them. We report for the first time, a novel *USP48* splice-site mutation (p.Pro433IVS + 2 T > A).*USP8*-mutated tumours had an increased presence of Crooke's hyaline change in the normal adenohypophysis. Tumours with *USP48* mutations had significantly more cavernous sinus invasion than *USP48* wild-type adenomas.

Declarations

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Disclosure

The findings of this study were presented at the annual conference of the Indian Society of Neuro-Oncology, 2021.

Author contributions

APA, AGC, RP, GC and HSA contributed to conception and design of the study. AGC operated on the patients in this study. GC supervised the histopathological examination of all tumours. DLB and RP did DNA sequencing of the tumor samples. HSA, SR, NT and NK were responsible for diagnosis of the patients with Cushing's disease and their medical management. APA collected patient information, performed statistical analysis and interpretation of data, and wrote the first draft of the manuscript. All authors critically reviewed the manuscript. All authors read and approved the final version of the manuscript.

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Compliance with ethical standards

Conflict of interest

The authors declare no competing interests.

Ethics approval

The study was approved by the Ethics Committee of our Institutional Review Board (IRB Min No: 9628, dated 01.09.2015). Informed consent was obtained from all adult patients. In the case of minors, assent was obtained from patients along with the informed consent of their parents / guardians.

Availability of data and material

On request

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Tables

Due to technical limitations, table 1 to 6 are only available as a download in the Supplemental Files section.

Figures

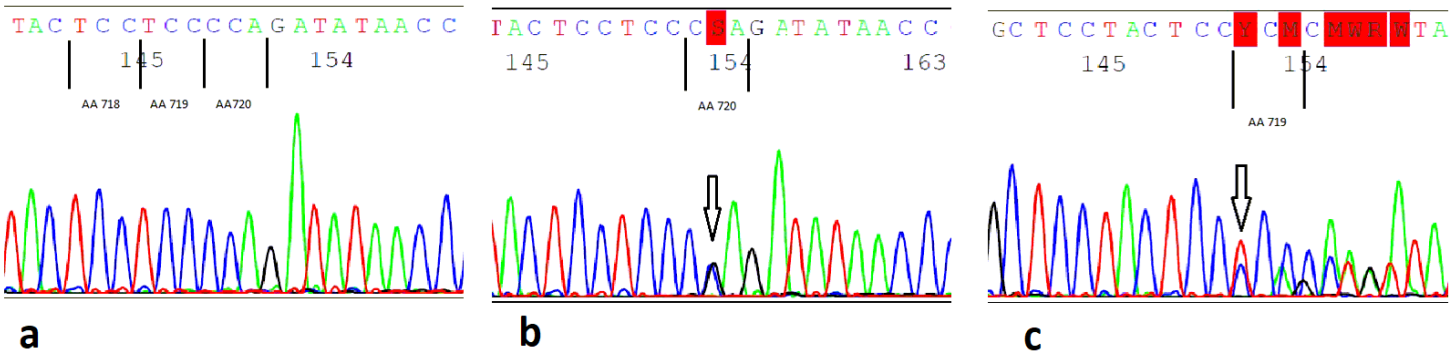

Figure 1

Fig. 1a - Sequencing chromatogram of a USP8 wild-type tumour Fig. 1b - Sequencing chromatogram showing a p.Pro720Arg mutation of USP8 Fig. 1c - Sequencing chromatogram showing a p.Ser719del fs mutation of USP8

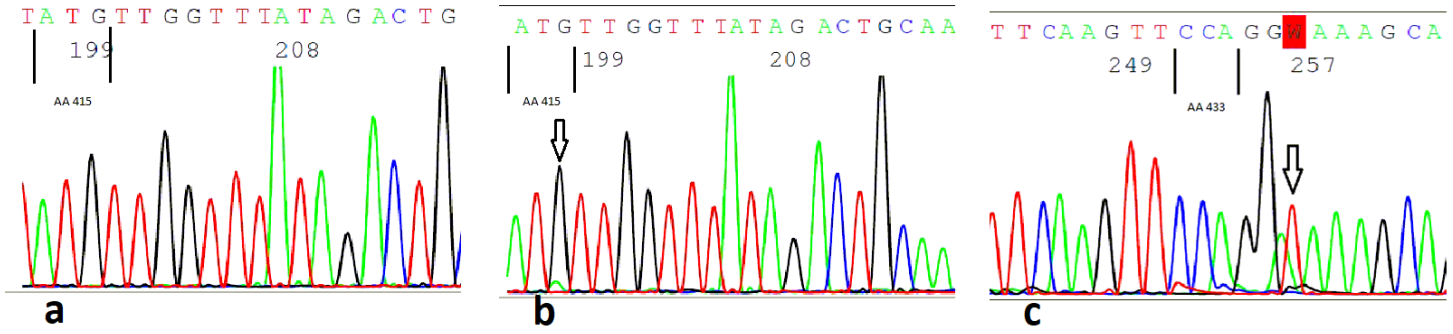

Figure 2

Fig. 2a - Sequencing chromatogram of USP48 wild-type tumour Fig. 2b - Sequencing chromatogram showing a p.Met415Ile mutation of USP48 Fig. 2c - Sequencing chromatogram showing a USP48 splice-site mutation (p.Pro433IVS+2 T>A)

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