High frequency of BRAF mutations in mucinous ovarian carcinoma of Taiwanese patients

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Abstract

Background

In view of the encouraging clinical evidence of *BRAF* inhibitors that can treat some melanoma patients successfully, we aimed to investigate the status of *BRAF* mutations of primary mucinous ovarian carcinomas (MOC) in Taiwanese women, and apply the emerging paradigm classification of BRAF mutation groups.

Methods

DNA was extracted from micro-dissected tissue samples using the QIAamp® DNA FFPE Kit. The mutations of activation segment (exon 15), CR3 (conserved regions 3), kinase domain of the *BRAF* gene were analyzed using the highly sensitive *BRAF* mutant enriched kit (FemtoPath®) with subsequent Sanger sequencing method. Additionally, we extended our prior data of *HER2* aberrations and *KRAS* mutation into this study in order to compare with the status of BRAF mutation.

Results

Out of the 20 cases tested, 16 (80%) harbored *BRAF* missense mutations. Their mutation profile and case number (n) were categorized as (1) class I: V600E (n = 1), V600M (n = 1); (2) class II: A598V (n = 1), T599I (n = 10); (3) class III: none (n = 0); and (4) unclassified variants: S602F (n = 2), dual T599I plus S602F (n = 1). The *BRAF* missense mutation (S602F) is novel. In addition, the prevalence of *BRAF* gene mutation is significantly higher than *HER2* gene mutation (80% vs. 35%; p = 0.022,) and *HER2* gene amplification (80% vs. 35%; p = 0.022), respectively. However, the mutation rates of *BRAF* and *KRAS* were not significantly different (80% vs. 60%; p = 0.289).

Discussion

Our results showed that *BRAF* mutation is not uncommon in primary MOC of Taiwanese. When taken together with previous published data, we found that the activating *BRAF* mutation, *HER2* amplification, *HER2* mutation and *KRAS* mutation were not mutually exclusive, but simultaneously independent. However, they may even have a synergistic effect in tumorigenesis.

Conclusions

The *BRAF* variant with T599I stands the majority. These findings suggested that there was a lower potential response to the existing V600 *BRAF* inhibitors, but may be responsive to dual *BRAF* plus *MEK* inhibitors or single *MEK* inhibitor in MOC. Further studies are warranted to investigate the clinical benefits

of newly targeted therapy in recurrent or advanced stage MOC patients carrying each class of *BRAF* mutation.

Background

After careful exclusion of metastatic diseases, primary mucinous ovarian carcinoma (MOC) comprised less than 3% of all epithelial ovarian cancers. For years, there has been much discussion that MOC seems to be a unique disease that responds poorly to conventional chemotherapy regimens. Even though long survival and favorable outcome can occur in general with early diagnosis and optimal operation, recurrent and advanced disease are associated with poor prognosis. So far, neither modern guidelines, nor therapeutic consensus existed for the best management of recurrent or advanced MOC. [1–3]

BRAF, a member of the rapidly accelerated fibrosarcoma (RAF) kinase family, is able to transduce signals downstream of *RAS* via the mitogen-activated protein kinase (MAPK) pathway. Under physiologic conditions, this pathway is tightly regulated through a negative feedback loop [4]. Activating mutations of *BRAF* can autonomously lead to uncontrolled cellular proliferation and cell survival. The anti-BRAF drugs are successfully used in clinical practice for melanoma and achieve favorable responses for low-grade serous ovarian carcinoma [5, 6]. Based on that information, we aimed to explore the *BRAF* mutation status and evaluate whether it can be a potential therapeutic target for patients with recurrent or advanced stage MOC.

Based on the mechanisms for activation of the MAPK pathway, a new model categorized the *BRAF* mutations into the following: (1) class I variants are V600 mutations, monomer with high level of kinase activity and *RAS* signaling independence, (2) class II variants are non-V600 mutations, dimer with intermediate to high level of kinase activity and *RAS* signaling independence, (3) class III variants are non-V600 mutations, dimer with absent or impaired kinase activity and *RAS* signaling dependence, and (4) unclassified variants are other *RAF* mutations that having unknown function. [7]

Currently, we not only investigated the mutational status of *BRAF* MOC in Taiwanese women, but also extended our prior data of *HER2* aberrations and *KRAS* mutation of all samples tested (n = 20) to this study. After that, we can (1) assess the pairwise comparison of *BRAF* mutation with the respective *KRAS* mutation, *HER2* mutation and *HER2* amplification; (2) evaluate the prevalence of *BRAF*-based dual, triple and quadruple mutation sets including the co-existing *BRAF* mutation with *KRAS* mutations, *HER2* mutations and *HER2* amplifications; (3) determine whether those MOC with wild-type *HER2* and *KRAS* can alternatively activate the MAPK signaling pathway through *BRAF* mutation; and (4) apply the emerging paradigm classification system for *BRAF* mutations and predict the potential implication of anti-*BRAF* therapeutic strategy in MOC.

Methods

Originally, we had 21 MOC tissue specimens left over from previous studies. However, except for one case missing residual DNA and lacking of enough extra tumor component, the characteristics of all remaining study materials of all 20 cases of MOC were described in our previous report, including tissue retrieval and DNA preparation. [2, 8–10] Additionally, 7 normal ovarian tissues are used as negative controls. The research was conducted according to International Conference on Harmonization guidelines and complied with all applicable regulations for protection of human subjects of research, including review and approval by the Institutional Review Board, Chung-Shan Medical University Hospital Taichung, Taiwan.

In this study, we used the FemtoPath *BRAF* Mutation Screen Kit, also named Medaysis Ultra-Sensitive *BRAF* Mutation Detection Kit. The Medaysis Inc. US is the delegation agent of the HongJing Inc. TW in the United States. Since the Medaysis business model should use its own brand in US, Femtopath's products need to be changed to Medaysis labels in their own brand names. This Kit applies a CloDiA™ PCR method by means of novel and proprietary mutation enrichment technology, in which two types of skills are involved - Unindel™ PCR and Stuntmer™ PCR. [11, 12] Unindel™ PCR is designed to identify a broad range of insertions/deletions (universal insertions/deletions) in the target region. Stuntmer™ PCR can detect a broad range of point mutations in sequence before and after V600 hot spots (amino acid range 591–620) in exons 15 of human *BRAF* gene. [13, 14]

Briefly, the sample nucleic acid with the mutation sequence is preferentially amplified over the wild type sequence by the self-competitive primers. PCR products were sequenced by the Sanger sequencing technique.

The pathogenicity associated with each *BRAF* mutations were identified in accordance with data of the Catalogue of Somatic Mutations in Cancer (COSMIC). The functional effect of novel *BRAF* missense mutations can be calculated using the Web software server Polymorphism Phenotyping v2 (PolyPhen-2), which can predict the possible impact of amino acid substitutions on the stability and function of human proteins using structural and comparative evolutionary considerations. [15]

McNemar's test was used to assess the significance of the difference between 2 paired dichotomous oncogenomic status, including (1) *BRAF* mutation rates vs. *KRAS* mutation rates; (2) *BRAF* mutation rates *vs. HER2* mutation rates; and (3) *BRAF* mutation rates vs. *HER2* amplification rates, individually. Data were analyzed using standard statistical software, version 9.0 (SPSS, Inc., Chicago, IL). The test was 2-sided and the significance level was 0.05.

Results

In this research, we found 4 cases (20%) with wild type *BRAF* and 16 cases (80%) with *BRAF* somatic missense mutations in all 20 cases tested. Of the 16 *BRAF* mutants, 1 had double missense mutations (T599I and S602F); 1 had a single missense mutation with an additional silent mutation (T599I and V600V); as well as 14 had single missense mutation including T599I (n = 9), A598V (n = 1), V600E (n = 1), V600M (n = 1) and S602F (n = 2). The missense mutation (S602F) is novel. All data of *BRAF* mutation

analysis were based on the Catalogue of Somatic Mutations in Cancer (COSMIC) database. The locations and types of *BRAF* mutations are presented in Table 1 and Fig. 1. Neither insertion, nor deletion of *BRAF* gene was detected. On the other hand, no BRAF mutations were detected in the 7 normal ovarian tissues. In addition, our previously published raw data of *HER2* amplifications, *HER2* mutations and *KRAS* mutations of all samples tested are integrated in Table 1.

Table 1
Oncogenic alteration analysis of MOC in twenty cases

Case number	Her2 gene mutation	Her2 gene amplified	KRAS gene mutation	BRAF gene mutation
1	Wild type	amplified	c.32C > T,p.A11V	c.1796C > T,p.T599I
2	c.2287G > A,p.A763T	Non-amplified	Wild type	c.1796C > T,p.T599I
3	c.2938G > A,p.A980T	Non-amplified	Wild type	c.1796C > T,p.T599I c.1800G > A,p.V600V
4	c.2329G > T,p.V777L	amplified	Wild type	c.1793C > T,p.A598V
5.	Wild type	Non-amplified	c.35G > A,p.G12D	c.1796C > T,p.T599I
6	Wild type	Non-amplified	c.35G > A,p.G12D	Wild type
7	Wild type	Non-amplified	c.35G > A,p.G12D	c.1796C > T,p.T599I
8	Wild type	Non-amplified	c.32C > T,p.A11V	c.1799T > A,p.V600E
9	Wild type	Non-amplified	c.35G > T,p.G12V	c.1796C > T,p.T599I
10	c.2555T > G,p.L852R	Non-amplified	c.35G > C,p.G12A	Wild type
11	c.2560A > G,p.K854E	Non-amplified	c.35G > T,p.G12V	c.1805C > T,p.S602F
12	Wild type	amplified	Wild type	c.1796C > T,p.T599I
13	Wild type	Non-amplified	c.35G > T,p.G12V	c.1796C > T,p.T599I
14	Wild type	Non-amplified	c.35G > A,p.G12D	c.1796C > T,p.T599I
15	Wild type	Non-amplified	c.35G > T,p.G12V	c.1796C > T,p.T599I c.1805C > T,p.S602F
16	Wild type	Non-amplified	Wild type	c.1798G > A,p.V600M

Case number	Her2 gene mutation	Her2 gene amplified	KRAS gene mutation	BRAF gene mutation
17	c.2908C > T,p.R970W	amplified	Wild type	Wild type
18	c.2912A > G,p.E971G	amplified	Wild type	c.1796C > T,p.T599I
19	Wild type	amplified	Wild type	Wild type
20	Wild type	amplified	c.32C > T, p.A11V c.37_38delinsAA,p.A13V c.40G > A, p.V14I	c.1805C > T,p.S602F

Using a pairwise comparison method, we identified that the prevalence of *BRAF* gene mutation is more common than that of *HER2* gene mutation (80% *vs.* 35%; p = 0.022, McNemar test). As well, the prevalence of *BRAF* gene mutation is more common than that *HER2* gene amplification (80% *vs.* 35%; p = 0.022, McNemar test). Even though the mutation rate of *BRAF* gene is numerically higher than that of *KRAS* gene, they both were not significantly different (80% *vs.* 60%; p = 0.289, McNemar test). (Fig. 2)

We identified that the dual set with *BRAF* and *KRAS* mutations occurred in 10 cases (50%), the dual set with *BRAF* mutation and *HER2* amplification occurred in 4 cases (20%), as well as the dual set with *BRAF* and *HER2* mutations occurred in in 5 cases (25%). Additionally, the triple set with *BRAF* mutation, *KRAS* mutation and *HER2* amplification occurred in 2 case (10%), the triple set with *BRA*F mutation, *KRAS* mutation and *HER2* mutation occurred in 1 case (5%), as well as the triple set with *BRAF* mutation, *HER2* mutation and *HER2* amplification occurred in 2 case (10%). Nonetheless, the quadruple set with *BRAF* mutation, *HER2* amplification, *HER2* mutation and *KRAS* mutation occurred in none (0%). (Table 1)

Adopting the newly classification scheme for *BRAF* mutations to MOC, we discovered 2 cases of class I *BRAF* mutants including V600E (n = 1) and V600M (n = 1); as well as 12 cases of class II *BRAF* mutants including A598V (n = 1), T599I (n = 10) and dual T599I/S602F (n = 1). No class III BRAF mutants were found. However, we identified 3 cases with the novel unclassified BRAF mutants, including single S602F (n = 2) and the dual T599I/S602F (n = 1) that are repeatedly counted. (Fig. 3)

Discussion

The *BRAF* (v-raf murine sarcoma viral oncogene homolog B1) gene is located on the long arm of chromosome 7 (7q34) and encodes for an 18-exon cytoplasmic protein, a serine/threonine protein kinase (B-Raf) which can be recruited to the membrane upon stimulation of *HER2* receptor. *BRAF* is a serine/threonine protein kinase, which is an important signal transducer of the *HER2* triggered *RAS*–*RAF*–mitogen-activated protein kinase kinase (*MEK*)–extracellular signal regulated kinase (*ERK*) signaling pathway (also known as *RAS/RAF/MEK/ERK* pathway or *MAPK* cascade). Active *BRAF* then activates

MEK1/2 to phosphorylate *ERK1/2*, which leads to the expression of several downstream transcription factors that regulate cell growth, differentiation, and survival. [4, 16]

We have already known that the early stage MOC usually has an excellent prognosis, but late stage MOC carries a poor outcome. [1] The encouraging success of *BRAF* inhibitors that can successfully treat some melanoma patients with *BRAF* (V600) mutations has prompted us to investigate the *BRAF* status and its therapeutic implication in advanced MOC. However, few studies have characterized its *BRAF* oncogene status and its response to anti-*BRAF* drugs has not yet been comprehensively explored in MOC. Even though melanoma and MOC are different tumors, we imagine that the functional consequence and anti-*BRAF* effect of the tumors harboring *BRAF* mutations might be similar. [17, 18]

After merging the previously reported raw data of *HER2* amplifications, *HER2* mutations and *KRAS* mutations with the new information of *BRAF*, we identified that their corresponding frequencies are 35% for *HER2* amplifications, 35% for *HER2* mutations, 60% for *KRAS* mutations and 80% for BRAF mutations in all 20 MOC Taiwanese patients (Table 1). [9, 10] Our findings also indicated that there was no significant difference in the frequency between BRAF mutations and KRAS mutations when they were compared. However, the frequency of *BRAF* mutations was significant higher than that of *HER2* amplifications and *HER2* mutations, respectively. (Fig. 2)

Focusing on the basis of *BRAF* variant together with *HER2* amplifications, *HER2* mutations, *KRAS* mutations within the *HER2* triggered MAPK signaling pathway, we divided them into the *BRAF*-based dual, triple and quadruple sets, respectively. After that, we identified that the *BRAF*-based dual and triple sets are not uncommon except for the quadruple set (Table 1). Our data indicated that the coexisting mutations of these driver genes indeed occur in MOC, which might cause synergistic effects in tumorigenesis.

In our study, one case showing *HER2* amplification, but wild-type *HER2*, *KRAS* and *BRAF* genes indicated that *HER2* alone may in fact confer heightened sensitivity to existing anti-HER2 therapies (i.e. trastuzumab, lapatinib) because of dependency on the most upstream receptor tyrosine kinase (RTK) signaling. (Table 1, case no. 19) Moreover, high frequency of *HER2* gene amplification (n = 7) combined with *HER2* mutation (n = 3; 42.86% & Table 1, case no. 4, 17, 18), with *KRAS* mutation (n = 2; 28.58% & Table 1, case no. 1, 20) or with *BRAF* mutation (n = 5; 71.43% & Table 1, case no. 1, 4, 12, 18, 20) can predict unresponsiveness or refractoriness to single *HER2* inhibition as a result of the constitutive activation of the MAPK pathway downstream of *HER2* signaling. On the other hand, we identified one case with *HER2* non-amplification, *HER2* wild-type, *KRAS* wild-type, but alternative *BRAF* mutation (V600M). (Table 1, case no. 16) It is suggested that the downstream *BRAF* (V600) mutations alone might be enough to trigger continuous activation of MAPK signaling cascade, leading to tumorigenesis. The existing V600 *BRAF* inhibitors (i.e. Vemurafenib, dabrafeniband and encorafenib) may have benefit to some Taiwanese patients with primary MOC; however, the clinical evidence that currently exists to substantiate these claims are insufficient.

Previous reports from other countries have shown that MOC has a lower frequency of BRAF mutation (2–20%). [1, 2, 6] Our data revealed that the *BRAF* missense mutation rate is relatively up to 80% (n = 16/20) using the FemtoPath BRAF Mutation Screen Kit. This kit is a PCR-based test using proprietary primers which can selectively amplify the somatic mutations in activating segment of the *BRAF* gene, and suppresses the amplification of wild-type *BRAF* gene in human genomic DNA. [13, 14] Although time-consuming, DNA sequencing techniques are still the current gold standard for mutational testing.

Despite geographical, racial and ethnic differences, the following 5 standpoints explain the reason why the *BRAF* mutation rate of this study is higher than that of others. (1) We used the H-E (hematoxylin and eosin stain) based microdissection technique to obtain a high percentage of representative tumor parts from formalin-fixed paraffin-embedded (FFPE) tissues, which restricted our analysis to only those tumor cells that express a specific marker or have a specific gene mutation. (2) According to the manufacture's manual and the previous report of the Stuntmer PCR technology, this highly specific and sensitive mutation enrich technology can detect less than 1% (as little as 20–100 ng) of *BRAF* V600 variants within exon 15. Additionally, the neighboring mutation sites of V600 (amino acid range 591–620) can also be amplified at the same time. Based on the identical principle of Stuntmer PCR, all of the *BRAF* mutations detected may share the similar high sensitivity. (3) We used other 7 normal ovarian tissues as negative controls, but none of them revealed *BRAF* mutations using the same kit. (4) Furthermore, the prior report also demonstrates that a stuntmer can inhibit wild type template replication, thereby allowing for selective amplification of mutants in a non-sequence specific manner. (5) Even after three rounds of PCRs, the original wild-type signal group remained unaltered, demonstrating that the stuntmer does not alter the original sequence of the sample [12–14]. The above-mentioned (1)-(5) evidences support that the possibility of false positive *BRAF* gene mutations detected using FemtoPath *BRAF* Mutation Screen Kit is extremely low.

According to the new classification system for *BRAF* mutations, different classes can predict their matching clinical response to contemporary targeted therapies on the market and have important implications for future anti-*BRAF* development. [7] In our patient cohort, we detected 4 kinds of known *BRAF* missense variants, 2 of which were class I (V600E, V600M), 2 were class II (A598V, T599I) and none was class III. Additionally, we identified one novel BRAF variant (S602F) that has never been reported in accordance with the COSMIC database. Even though the biochemical and signaling mechanism of the new *BRAF* variant (S602F) has not yet been comprehensively studied, its predicted functional effect appeared to be probably damaging in accordance with the Polyphen-2 database. As well, we suspect that the S602F might be categorized as class II BRAF variant, because it is located in the activating segment of *BRAF* kinase domain.

In summary, *BRAF* mutation is not uncommon in primary MOC of Taiwanese. When taken together with previous published data, we found that the activating *BRAF* mutation, *HER2* amplification, *HER2* mutation and *KRAS* mutation were not mutually exclusive, but simultaneously independent. However, they may even have a synergistic effect in tumorigenesis. Even though our results are confident and

comprehensive, the case number cohort was small. Further exploratory studies should be performed to validate these finding.

Conclusions

Despite possible underestimation in some other studies, our solid data demonstrated that *BRAF* gene mutation rate was 80% of primary MOC in Taiwanese patients, using the FemtoPath *BRAF* Mutation Screen Kit. One novel BRAF mutation (S602F) has been identified in this study. Unlike melanoma and papillary thyroid carcinoma, the most common BRAF mutation of MOC is the non-V600 class II variant with T599I (n = 11/20; 55%) in Taiwanese. So far, there are no effective targeted treatments available for patients who have tumors or diseases harboring non-V600 *BRAF* mutations. [5, 19] Our results highlight the importance of developing new anti-*BRAF* therapeutic options for such patients harboring non-V600 *BRAF* mutations. Alternatively, it indicated that the potential treatment strategy of recurrent or metastatic MOC might favor dual *BRAF* plus *MEK* inhibitor or single MEK inhibitor rather than the existing anti-BRAF V600 class I inhibitors. Further studies are warranted to investigate the clinical benefits of class-specific therapy in *BRAF*-altered metastatic or advanced stage MOC patients.

Abbreviations

BRAF
Proto-oncogene B-Raf and v-Raf murine sarcoma viral oncogene homolog B
Kras
V-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog gene
Her2
Human epidermal growth factor receptor 2 gene
MAPK
Mitogen-Activated Protein Kinases

Declarations

1. Ethics approval and consent to participate:

Our research was conducted in accordance with the International Conference on Harmonization (ICH) guidelines and compliant with all applicable regulations and the protection of human subjects for research, including review and approval by the Institutional Review Board of the Chung-Shan Medical University Hospital, Taichung, Taiwan.

2. Consent for publication:

Not applicable.

3. Availability of data and material:

Please contact author for data requests.

4. Competing interests:

The authors declare that there are no competing interests.

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6. Authors' contributions:

CPH and GTH conceived and designed the research; YJL, WRC performed the experiments and wrote the manuscript. All authors read and approved the final manuscript.

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Figures

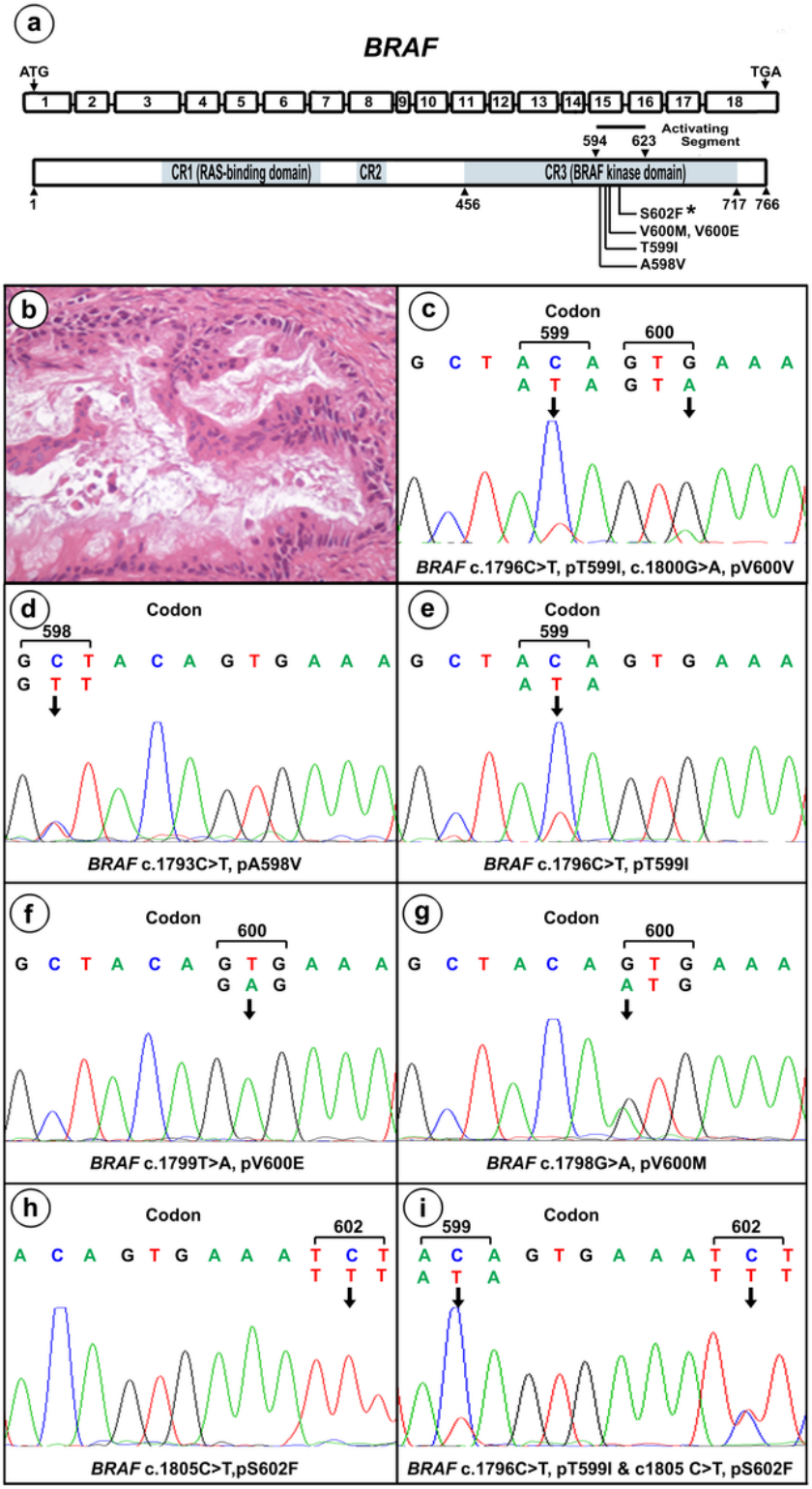

Figure 1

(a) BRAF is located on chromosome 7q34 and contains 18 exons with intervening sequences. The start codon (ATG) and stop codon (TGA) are indicated. Schematic drawings show that the detected BRAF missense mutations are located within the activation segment (exon 15) of the CR3 (conserved regions 3), kinase domain in MOC. An asterisk (*) indicates the novel finding in this report, not documented in the COSMIC database. (b) Representative case showing histopathologic picture with Hematoxylin-eosin

stain, 400x. (c), (d), (e), (f), (g), (h), (i) DNA electropherograms depicting the various BRAF misense mutations by arrows, and validating by direct Sanger sequencing.

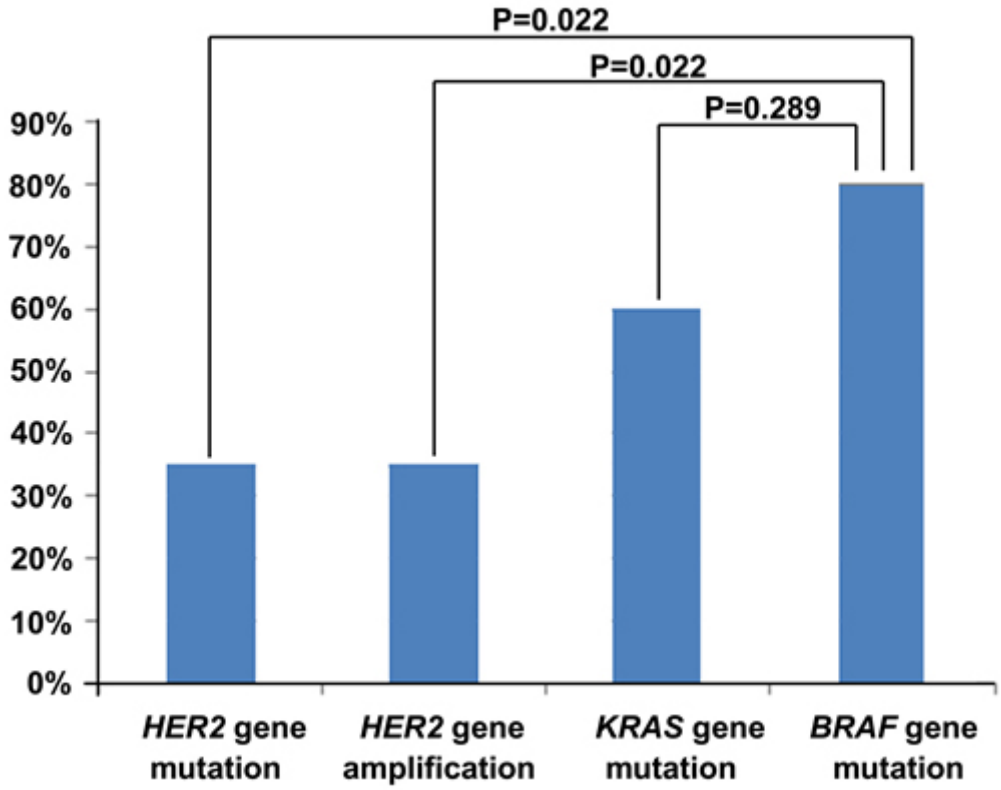

Figure 2

Pairwise comparison of frequencies of BRAF gene mutation vs. KRAS gene mutation; BRAF gene mutation vs. HER2 gene mutation; and BRAF gene mutation vs. HER2 gene amplification using McNemar test (α = 0.05).

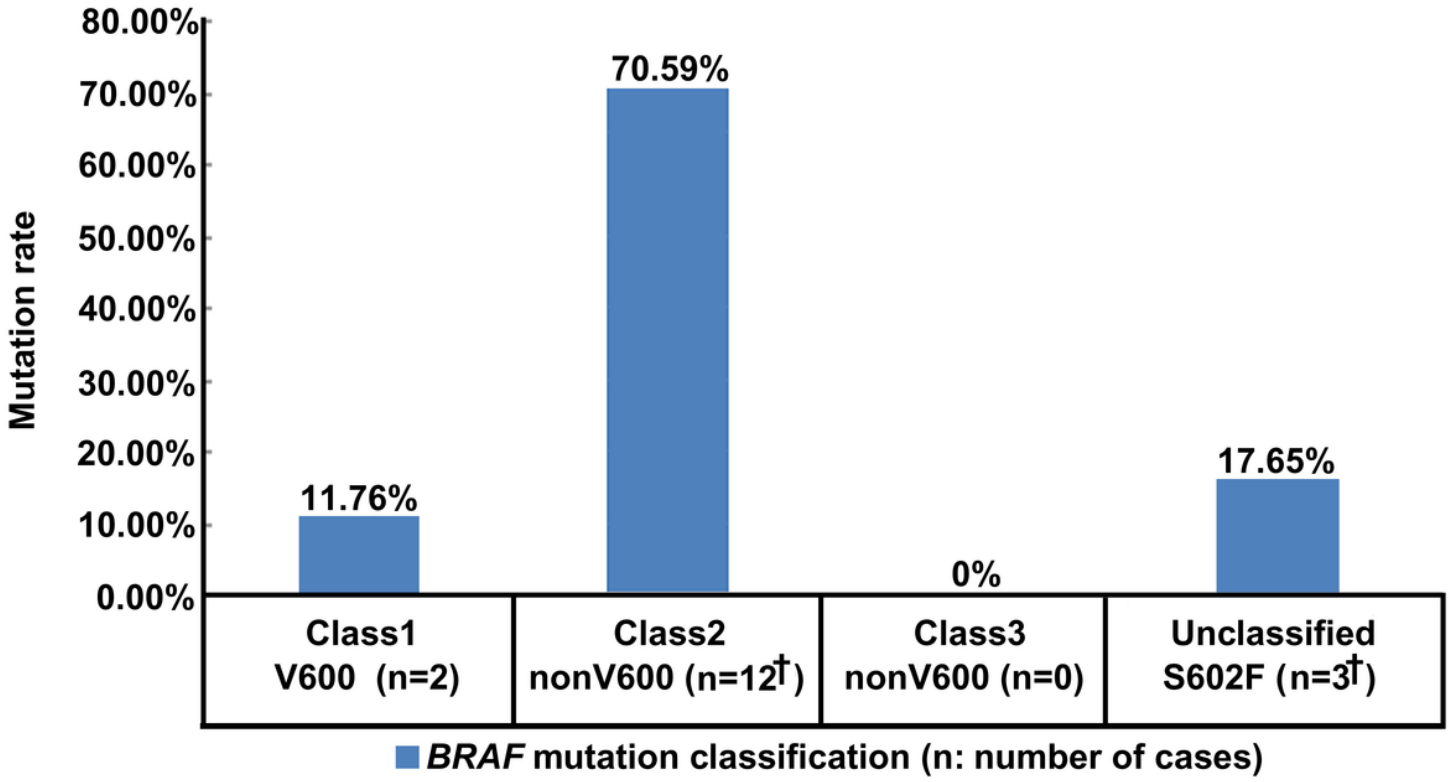

Figure 3

legend BRAF mutations are categorized into class-1 (high kinase activity, V600), class-2 (high or intermediate kinase activity, nonV600), class-3 (impaired BRAF kinase activity) and unclassified (unknown BRAF kinase activity). The “n” means number of cases existed. The symbol “†”means one case with dual missense mutations (T599I and S602F) of BRAF that are counted twice in class 2 and unclassified categories, disparately.