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Brief Report

The absence of the *ERBB4* hotspot mutations in melanomas in patients from southern China

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Abstract

V-erb-a erythroblastic leukemia viral oncogene homolog 4 (*ERBB4*) has been reported to be somatically mutated in 19% of melanoma cases. To investigate the prevalence of *ERBB4* mutations in melanoma patients from southern China, we analyzed 117 formalin-fixed, paraffin-embedded melanoma samples archived in the Sun Yat-sen University Cancer Center. A matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) platform was used to screen for mutations. No *ERBB4* hotspot mutations were detected. Our results indicate that *ERBB4* mutations may play a limited role in melanomas in China; therefore, targeting the *ERBB4* mutation in melanoma patients from southern China may not be a promising strategy.

Key words Melanoma, *ERBB4*, mutation, Chinese

Malignant melanomas are aggressive and highly resistant to conventional chemotherapy; therefore, less toxic, targeted melanoma treatments are needed. V-raf murine sarcoma viral oncogene homolog B1 (*BRAF*) mutations have been reported in approximately 40% to 60% of cutaneous melanomas[1]. Treatments with vemurafenib, a *BRAF* kinase inhibitor, resulted in improved rates of overall and progression-free survival compared with treatments with dacarbazine in patients with previously untreated melanoma expressing the *BRAF* V600E mutation[2]. Although these findings appear promising, a much larger pool of both potential targets and effective therapeutic strategies is undoubtedly required. Recently, Prickett *et al.*[3] reported that somatic mutations in *ERBB4* are common in malignant melanomas, and these researchers provided compelling evidence that mutated *ERBB4* is a promising novel drug target for metastatic melanoma. The identification of these mutations would be an important step in understanding the mechanisms underlying the development of malignant melanoma. Dutton-Regester *et al.*[4] observed that *ERBB4* mutations occurred only in 2% of melanoma cases in Australia. The prevalence of mutations in melanoma susceptibility genes may vary among different geographic areas[5]. Therefore, this study was undertaken to evaluate the presence of *ERBB4* mutations in melanoma patients from southern China.

Materials and Methods

In total, 117 archival formalin-fixed, paraffin-embedded melanoma samples were collected for mutation analysis. This study was conducted in accordance with the recommendations of the Declaration of Helsinki and was approved by the local ethics committees of the Sun Yat-sen University Cancer Center, China. Signed informed consent was obtained from all subjects to allow the use of their samples and records for research.

DNA was isolated from the melanoma samples using the QIAamp DNA FFPE Tissue Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. The quality of the isolated DNA was assessed using the absorbance 260/280 ratio and 1% agarose gel electrophoresis. For mutation screening, the MALDI-TOF MS platform (Sequenom, San Diego, CA) was used according to the protocol provided by Sequenom. Nineteen assays interrogating hotspot mutations in ERBB4 were designed on the basis of the report by Prickett *et al.*[3]. Both PCR primers and MassEXTEND primers for multiplexed assays were designed using Sequenom's MassARRAY Assay Design software, v3.1 (Sequenom). The assay design and the list of amplification and extension primers are provided in Tables 1 and 2.

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Genomic DNA was amplified using the designed PCR primers, unincorporated nucleotides were inactivated by shrimp alkaline phosphatase (SAP), and a single base extension reaction was performed using the extension primers. Salts were removed through the addition of Clean Resin (Sequenom), and the multiplexed reaction solution was dispensed onto a 384-sample SpectroCHIP II matrix chip

Table 1. Assay design for amplification and extension primers

Mutation ID	Amplicon length	Ext. primer direction	Ext. primer mass	First allele call	First allele mass	Second allele call	Second allele mass
E452K	156	R	4,524.9	G	4,772.1	A	4,852.0
E563K	142	R	4,937.2	G	5,184.4	A	5,264.3
P409L	152	F	5,139.4	C	5,386.5	T	5,466.5
E836K	162	R	5,529.6	G	5,776.8	A	5,856.7
M313I	137	R	5,943.9	G	6,191.1	A	6,271.0
E872K	151	R	6,048.0	G	6,295.1	A	6,375.0
Y111H	148	F	4,905.2	C	5,152.4	T	5,232.3
P700S	151	F	5,209.4	C	5,456.6	T	5,536.5
E542K	139	R	5,402.5	G	5,649.7	A	5,729.6
L39F	151	R	5,563.6	T	5,834.8	C	5,850.8
E317K	137	R	6,913.5	G	7,160.7	A	7,240.6
P1033S	163	F	4,736.1	C	4,983.3	T	5,063.2
R1174Q	144	F	5,744.7	A	6,016.0	G	6,032.0
R393W	150	F	5,835.8	C	6,083.0	T	6,162.9
R544W	139	F	6,441.2	C	6,688.4	T	6,768.3
S341L	144	F	4,301.8	C	4,549.0	T	4,628.9
D609N	136	R	4,528.0	G	4,775.1	A	4,855.1
G936R	136	R	4,881.2	G	5,128.4	A	5,208.3
R491K	159	R	6,625.3	G	6,872.5	A	6,952.4

Table 2. Primers used in amplification and extension experiments for the detection of *ERBB4*

Mutation ID	First PCR primer	Second PCR primer	Extension primer
E452K	5′-ACGTTGGATGCTTATCCTCAAGCAACAGGG-3′	5′-ACGTTGGATGTGCTGAAGAGTGTTGTCCAG-3′	5′-TTCCTGCGCTGATTT-3′
E563K	5′-ACGTTGGATGTTCGGGAGTTTGAGAATGGC-3′	5′-ACGTTGGATGAAGCCAACACACCACAGATG-3′	5′-GTGAGGAGGCCATCTT-3′
P409L	5′-ACGTTGGATGTGAGCCCTGCAGCTTTAAAC-3′	5′-ACGTTGGATGCACTTGTAGGTTTCCTGAAC-3′	5′-CATACAGTCATGGCCAC-3′
E836K	5′-ACGTTGGATGTAGGCACTTCCAACTGAAGG-3′	5′-ACGTTGGATGCAAATCCCGATGAACGAGTC-3′	5′-GATGAACGAGTCGTCTTT-3′
M313I	5′-ACGTTGGATGTTCCAGTTCTTGTGTGCGTG-3′	5′-ACGTTGGATGAATCTGAGCTACCACTCACC-3′	5′-ATCCCATTTTCTTCTACTTC-3′
E872K	5′-ACGTTGGATGACTCGTTCATCGGGATTTGG-3′	5′-ACGTTGGATGCCTTTCCTCCATCAGCATTG-3′	5′-ATCAGCATTGTACTCTTTTT-3′
Y111H	5′-ACGTTGGATGAATATTGCCAAGGCATATCG-3′	5′-ACGTTGGATGCAGTCTGTTCGAGAAGTCAC-3′	5′-TCGTGGGACAAAACTT-3′
P700S	5′-ACGTTGGATGAACCGTTCCAAAAGCACCTG-3′	5′-ACGTTGGATGTTTCCGCTTTGCAGTTGGTG-3′	5′-TGGTGGAACCATTAACT-3′
E542K	5′-ACGTTGGATGGACGCTTGTTTGCTTACACC-3′	5′-ACGTTGGATGGCCATCTTCCATCTTCTCAC-3′	5′-TCTCAAACTCCCGAAATT-3′
L39F	5′-ACGTTGGATGTGTCTTTGCAGTGTGTGCAG-3′	5′-ACGTTGGATGTGCTGGTTATCTCCAGGTTG-3′	5′-TGTTCCAGGTCAGAGAGA-3′
E317K	5′-ACGTTGGATGTTCCAGTTCTTGTGTGCGTG-3′	5′-ACGTTGGATGAATCTGAGCTACCACTCACC-3′	5′-TTTACACATTTTAATCCCATTTT-3′
P1033S	5′-ACGTTGGATGCGAGTCAATTCTTGCTCTGG-3′	5′-ACGTTGGATGCCAGTCCAAATGACAGCAAG-3′	5′-TTTCAACATCCCACCT-3′
R1174Q	5′-ACGTTGGATGTTCACATACTCATCCTCGGC-3′	5′-ACGTTGGATGTGAATCCAGTGGAGGAGAAC-3′	5′-GAGAACCCTTTTGTTTCTC-3′
R393W	5′-ACGTTGGATGGATGCCCAGTCAATCTTGTG-3′	5′-ACGTTGGATGAGCCATAGACCCAGAGAAAC-3′	5′-AGAGAAACTGAACGTCTTT-3′
R544W	5′-ACGTTGGATGGCCATCTTCCATCTTCTCAC-3′	5′-ACGTTGGATGGACGCTTGTTTGCTTACACC-3′	5′-TGATGTTTTCACAGTGAATTT-3′
S341L	5′-ACGTTGGATGAATCTGAGCTACCACTCACC-3′	5′-ACGTTGGATGGTGGTAGATTCCAGTTCTTG-3′	5′-TGTGTGCGTGCCTG-3′
D609N	5′-ACGTTGGATGATGTCCAGATGGCTTACAGG-3′	5′-ACGTTGGATGTGGCCAGCAAGAATGCTTAC-3′	5′-ACTCCCGATCTGGAT-3′
G936R	5′-ACGTTGGATGTGGGAACTGATGACCTTTGG-3′	5′-3′ACGTTGGATGACGTCAATAGTGCAGATGGG-3′	5′-TGAGGCAAACGTTCTC-3′
R491K	5′-ACGTTGGATGACTGGACAACACTCTTCAGC-3′	5′-3′ACGTTGGATGTTGGTCCAAAGAAGAATGGG-3′	5′-CTTACTACAATTTTCAGCTTTT-3′

PCR, polymerase chain reaction.

using the MassARRAY Nanodispenser RS1000 (Sequenom). Mass spectrometry analysis was performed using a matrix-assisted laser desorption/ionization time-of-flight mass spectrometer (Sequenom), and the resulting data were analyzed with the MassARRAY Typer Analyzer software, v4.0.4.20 (Sequenom). The Sequenom software produces genotype performance grades ranging from "conservative" (high quality) to "moderate" (intermediate quality) to "aggressive" (low quality). The "user call" designation indicates manual genotyping, whereas the "no call" annotation implies the failure of automated genotyping.

Results

Among the 117 melanoma samples, there were 48 acral melanomas, 36 mucosal melanomas, and 33 melanomas on the skin without chronic sun-induced damage (non-CSD melanomas). The median age of the 117 patients was 54 years (range, 29–82 years). The characteristics of these patients are listed in Table 3. No *ERBB4* hotspot mutations were detected in the examined melanoma cases by MALDI-TOF MS. To confirm these results, we repeated the experiments and found that the data were consistent. To demonstrate the sensitivity and the specificity of the MALDI-TOF MS platform, we performed a plasmid mixing experiment. A pCMV6-XL6 plasmid containing the *ERBB4* K751M mutation was purchased from OriGene Technologies, Inc. (Rockville, USA). The wild-type clone of *ERBB4* was generated from the corresponding mutant clone by site-directed mutagenesis. Mutation analysis was performed using either the pCMV6-XL6 alone or mixtures of the pCMV6-XL6 plasmid with various percentages of the wild-type *ERBB4* clone. The mutation was detectable for samples in which the *ERBB4* K751M plasmid represented only 5% to 10% of the total DNA (Figure 1). This level of sensitivity is important for the detection of mutations in clinical cancer samples, which usually contain normal tissue that dilutes the proportion of tumor cells in each sample.

Discussion

ERBB4 (HER4) is a member of the ERBB family of receptor tyrosine kinases (RTKs); this family also includes the epidermal growth factor receptors (*EGFR/ERBB1/HER1*, *ERBB2/HER2/Neu*, and *ERBB3/HER3*)[6]. All members of this family have the same structure, consisting of an extracellular ligand-binding domain, a transmembrane domain, and a cytoplasmic protein tyrosine kinase domain. The aberrant activation of *ERBB1/EGFR* and *ERBB2/HER2* contributes significantly to neoplastic formation, progression, and proliferation[7]. Accordingly, these proteins are considered to be promising candidates for tumor-targeted therapy. However, the biological role of *ERBB4* and its potential applicability as a cancer drug target remain unclear. Several studies suggest that *ERBB4* induces growth inhibition or apoptosis[8]; however, *ERBB4* has also been documented to promote proliferation and tumor growth[9,10]. These discrepancies may be related to the alternative splicing of the *ERBB4* mRNA, which produces isoforms that may have distinct functions. Prickett *et al.*[3] demonstrated the involvement of *ERBB4* in the development of cutaneous metastatic melanoma harboring these mutations. *ERBB4* was found to be somatically mutated

Table 3. Demographics and clinical characteristics of melanoma patients from southern China

Variable	No. of patients (%)
Gender	
Female	52 (44.4)
Male	65 (55.6)
Tumor type	
Acral	48 (41.0)
Mucosal	36 (30.8)
Non-CSD	33 (28.2)
TNM stage	
I	6 (5.1)
II	27 (23.1)
III	56 (47.9)
IV	28 (23.9)
Ulceration	
Absent	56 (47.9)
Present	61 (52.1)

CSD, chronic sun-induced damage.

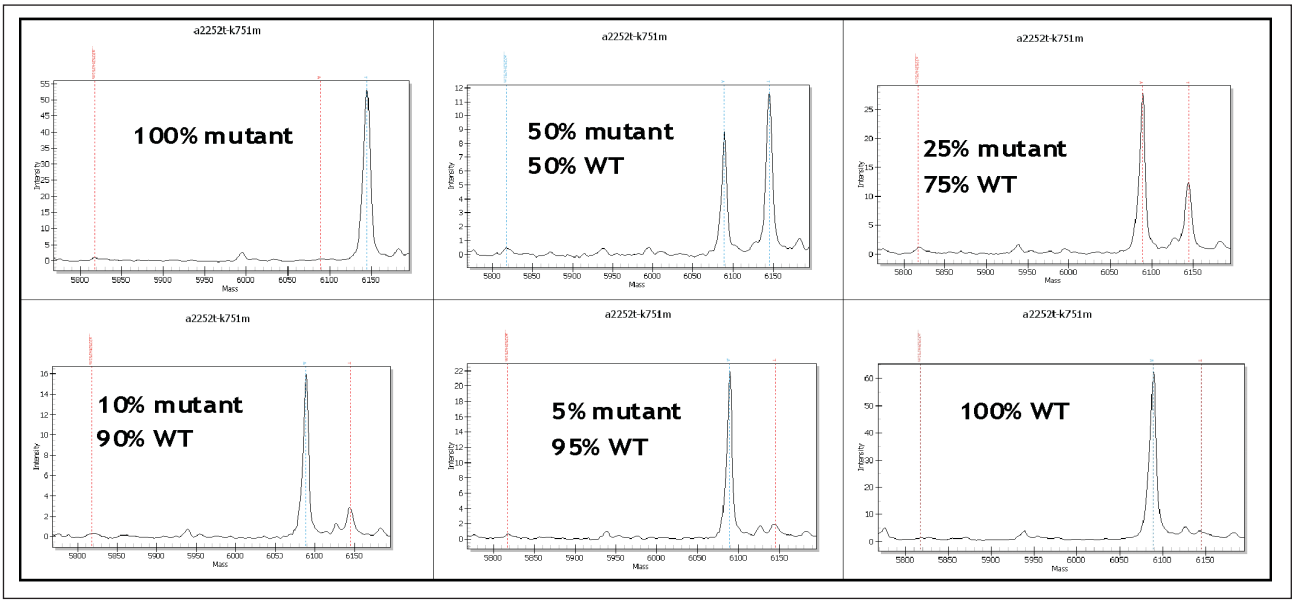

Figure 1. **The quantification of the sensitivity using a plasmid mixing experiment.** Spectra of the pCMV6-XL6 (mutant) alone and mixtures of the pCMV6-XL6 with the wild-type (WT) clone are shown. The provided percentages are based on the quantities of DNA (in ng) in each sample. This assay detected a K751M mutation in ERBB4.

in 19% of the examined melanoma cases. The functional analysis of 7 *ERBB4* mutants revealed that these mutations increase the protein's catalytic and transformation abilities and provide essential survival signals. These findings reveal a potentially important therapeutic opportunity for this challenging disease.

A high incidence of *ERBB4* mutation in malignant melanomas in Western populations inspired us to determine the prevalence of *ERBB4* mutations in Chinese melanoma patients. However, the present study indicates that hotspot mutations in the *ERBB4* gene are rare in Chinese patients with melanomas, suggesting that these mutations play a limited role in these patients. Because MALDI-TOF MS can only detect the mutations that have already been reported, our results cannot exclude the possibility that specific mutations of *ERBB4* exist only in Chinese melanoma patients.

Conclusions

Mutations in *ERBB* family genes may be important in cancer research because they are not only involved in tumorigenesis but also targeted in cancer therapy. However, our current data did not identify any significant prevalence of *ERBB4* hotspot mutations in melanoma patients from southern China, suggesting that targeting *ERBB4* hotspot mutations in melanoma patients from southern China might not be a promising therapeutic strategy for this disease.

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