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

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. 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.
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            |
| R395W       | 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                  | 4,629.9            |
| D609N       | 136             | R                     | 4,528.0          | G                | 4,775.1           | A                  | 4,851.5            |
| G939R       | 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'-ACGTTGATGCTTTATCCCTCAAGCAAACGGG-3' | 5'-ACGTTGATGCTTTATCCCTCAAGCAAACGGG-3' | 5'-TTCTCGCCGCTTATTT-3' |
| E563K       | 5'-ACGTTGATGCTTTATCCCTCAAGCAAACGGG-3' | 5'-ACGTTGATGCTTTATCCCTCAAGCAAACGGG-3' | 5'-GTGAGGAGGCCATT-3' |
| P409L       | 5'-ACGTTGATGCTTTATCCCTCAAGCAAACGGG-3' | 5'-ACGTTGATGCTTTATCCCTCAAGCAAACGGG-3' | 5'-ACGTTGATGCTTTATCCCTCAAGCAAACGGG-3' |
| E836K       | 5'-ACGTTGATGCTTTATCCCTCAAGCAAACGGG-3' | 5'-ACGTTGATGCTTTATCCCTCAAGCAAACGGG-3' | 5'-ACGTTGATGCTTTATCCCTCAAGCAAACGGG-3' |
| M313I       | 5'-ACGTTGATGCTTTATCCCTCAAGCAAACGGG-3' | 5'-ACGTTGATGCTTTATCCCTCAAGCAAACGGG-3' | 5'-ACGTTGATGCTTTATCCCTCAAGCAAACGGG-3' |
| E872K       | 5'-ACGTTGATGCTTTATCCCTCAAGCAAACGGG-3' | 5'-ACGTTGATGCTTTATCCCTCAAGCAAACGGG-3' | 5'-ACGTTGATGCTTTATCCCTCAAGCAAACGGG-3' |
| Y111H       | 5'-ACGTTGATGCTTTATCCCTCAAGCAAACGGG-3' | 5'-ACGTTGATGCTTTATCCCTCAAGCAAACGGG-3' | 5'-ACGTTGATGCTTTATCCCTCAAGCAAACGGG-3' |
| P700S       | 5'-ACGTTGATGCTTTATCCCTCAAGCAAACGGG-3' | 5'-ACGTTGATGCTTTATCCCTCAAGCAAACGGG-3' | 5'-ACGTTGATGCTTTATCCCTCAAGCAAACGGG-3' |
| E542K       | 5'-ACGTTGATGCTTTATCCCTCAAGCAAACGGG-3' | 5'-ACGTTGATGCTTTATCCCTCAAGCAAACGGG-3' | 5'-ACGTTGATGCTTTATCCCTCAAGCAAACGGG-3' |
| L39F        | 5'-ACGTTGATGCTTTATCCCTCAAGCAAACGGG-3' | 5'-ACGTTGATGCTTTATCCCTCAAGCAAACGGG-3' | 5'-ACGTTGATGCTTTATCCCTCAAGCAAACGGG-3' |
| E317K       | 5'-ACGTTGATGCTTTATCCCTCAAGCAAACGGG-3' | 5'-ACGTTGATGCTTTATCCCTCAAGCAAACGGG-3' | 5'-ACGTTGATGCTTTATCCCTCAAGCAAACGGG-3' |
| P1033S      | 5'-ACGTTGATGCTTTATCCCTCAAGCAAACGGG-3' | 5'-ACGTTGATGCTTTATCCCTCAAGCAAACGGG-3' | 5'-ACGTTGATGCTTTATCCCTCAAGCAAACGGG-3' |
| R1174Q      | 5'-ACGTTGATGCTTTATCCCTCAAGCAAACGGG-3' | 5'-ACGTTGATGCTTTATCCCTCAAGCAAACGGG-3' | 5'-ACGTTGATGCTTTATCCCTCAAGCAAACGGG-3' |
| R395W       | 5'-ACGTTGATGCTTTATCCCTCAAGCAAACGGG-3' | 5'-ACGTTGATGCTTTATCCCTCAAGCAAACGGG-3' | 5'-ACGTTGATGCTTTATCCCTCAAGCAAACGGG-3' |
| R544W       | 5'-ACGTTGATGCTTTATCCCTCAAGCAAACGGG-3' | 5'-ACGTTGATGCTTTATCCCTCAAGCAAACGGG-3' | 5'-ACGTTGATGCTTTATCCCTCAAGCAAACGGG-3' |
| S341L       | 5'-ACGTTGATGCTTTATCCCTCAAGCAAACGGG-3' | 5'-ACGTTGATGCTTTATCCCTCAAGCAAACGGG-3' | 5'-ACGTTGATGCTTTATCCCTCAAGCAAACGGG-3' |
| D609N       | 5'-ACGTTGATGCTTTATCCCTCAAGCAAACGGG-3' | 5'-ACGTTGATGCTTTATCCCTCAAGCAAACGGG-3' | 5'-ACGTTGATGCTTTATCCCTCAAGCAAACGGG-3' |
| G939R       | 5'-ACGTTGATGCTTTATCCCTCAAGCAAACGGG-3' | 5'-ACGTTGATGCTTTATCCCTCAAGCAAACGGG-3' | 5'-ACGTTGATGCTTTATCCCTCAAGCAAACGGG-3' |
| R491K       | 5'-ACGTTGATGCTTTATCCCTCAAGCAAACGGG-3' | 5'-ACGTTGATGCTTTATCCCTCAAGCAAACGGG-3' | 5'-ACGTTGATGCTTTATCCCTCAAGCAAACGGG-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 \textit{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 \textit{ERBB4} K751M mutation was purchased from OriGene Technologies, Inc. (Rockville, USA). The wild-type clone of \textit{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 \textit{ERBB4} clone. The mutation was detectable for samples in which the \textit{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

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