Whole-exome sequencing detection of the somatic mutations associated with the tumorigenesis and gefitinib-response of mucoepidermoid carcinomas

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Abstract

Background: We performed whole-exome sequencing (WES) on the sputum and blood samples of a MEC patient exploring the genetic alternations underlying the mechanism of mucoepidermoid carcinomas (MEC) and gefitinib response.

Methods: We previously reported a 10-year old MEC patient who was cured after a complete response to gefitinib treatment. Whole-exome sequencing (WES) was performed on the samples of this patient to detect somatic mutations. Detected genes harboring somatic mutations were compared with previously reported mutant genes related to MEC.

Results: Somatic mutations were detected in 13 previously reported oncogene and tumor suppressors, and enriched in apoptosis (*RIPK1*, *SPTA1*, and *ACTG1*). The loss and gain of phosphorylation amino acids occurred in 8 of the 34 non-synonymous mutations, which resided in *ARL6*, *DNAH11*, *PGM5*, *PRAMEF15*, *RALGAPB*, *RANBP2*, *TTN*, and *UBN1*. *TTN* had two Ala to Thr mutations. Among the 50 genes containing detected somatic mutations, *ADAM28*, *DYSF*, *GP2*, *PPP2R5B*, and *TTN* were also detected in a previous study; and all of these overlaps were identified in low and intermediate grade samples.

Conclusions: These findings underline the possibility of the accumulated somatic mutations in the tumor suppressor genes and oncogenes might contribute to the tumorigenesis of our MEC patient, which have potential applications for the therapies of MEC.

Background

Mucoepidermoid carcinoma (MEC) is the most common malignant neoplasm arising from the salivary gland (Boukheris, Curtis et al. 2009, Speight and Barrett 2010). MEC usually derives from major glands, such as parotid and submandibular, or from the minor salivary glands lining oral cavity and the tracheobronchial tree. MEC is characterized by its cellular heterogeneity and consists of mucin-producing, epidermoid, and intermediate cells, which is defined as an uncommon tumor (Colby and Koss 1995). According to the WHO classification system, MECs are classified as low-, intermediate- or high-grade based on five histological features: the presence of a cystic component, neural invasion, necrosis, mitotic activity and anaplasia (Thompson 2006).

The previous traditional genetic studies have revealed some mechanisms of the etiology of MEC. About 35% (6 of 17) of high grade MECs harbored H-RAS mutations at codons 12, which strongly correlated with tumor grade (Yoo and Robinson 2015). High-grade MECs harbored an increased EGFR gene copy number (Lujan, Hakim et al. 2010) and mutations within the EGFR pathway and other key oncogenes (Cros, Sbidian et al. 2013). Particularly, the translocation of chromosomes 11q and 19p has been proposed as an early event of MEC tumorigenesis (Nordkvist, Gustafsson et al. 1994, El-Naggar, Lovell et al. 1996, Kang, Tan et al. 2016). This translocation, reported in approximately 30–80% of MECs tumors, produces fusion between the genes CREB-regulated transcriptional co-activator 1 (CRTC1; also known as

MECT1, TORC1 and WAMTP1) at 19p13 and Mastermind-like protein 2 (MAML2, a Notch receptor-induced transcription co-activator) at 11q21 (Nordkvist, Gustafsson et al. 1994, Behboudi, Enlund et al. 2006, O'Neill 2009, Seethala, Sanja et al. 2010, Chiosea, Dacic et al. 2012, Bell 2013). The resulted fusion protein causes disruption of cell-cycle regulation and differentiation (Tonon, Modi et al. 2003). Copy-number variations (CNVs) have been found to occur more frequently in fusion-negative MECs, with potential loss of tumor suppressor genes, such as DCC, SMAD4, GALR1, and CDKN2A/B, and gain of oncogenes, such as MAFA, LYN, MOS, and PLAG1 (Jee, Persson et al. 2013).

To date, only one whole-exome sequencing (WES) study has been performed on 18 MEC patients to globally characterized the somatic mutations contributing the etiology of MEC, showing that TP53 was the most commonly mutated gene in MEC (28%), and mutations were found only in intermediate- and high- grade tumors. Only 8 other genes (POU6F2 IRAK1, MAP3K9, ITGAL, ERBB4, OTOGL, KMT2C, and OBSCN) carried somatic alterations in at least two patients (Kang, Tan et al. 2016).

In vitro and clinical data have indicated that pulmonary MECs are responsive to gefitinib, the inhibitor of the tyrosine kinase activity of the epidermal growth factor receptor (EGFR). Sensitizing EGFR mutations (exon 19 deletions or exon 21[L858R] mutations) importantly affect the therapeutic response to tyrosine kinase inhibitors (TKIs) (Sharma, Bell et al. 2007). However, several studies have reported a clinical response to gefitinib in MECs lacking sensitizing EGFR mutations (Han, Kim et al. 2008, Rossi, Sartori et al. 2009). One explanation for the observed sensitivity to gefitinib in these cases may be upregulation of the EGFR ligand amphiregulin (AREG) by CRTC1-MAML2 (Coxon, Rozenblum et al. 2005). We have recently shown that gefitinib inhibited the phosphorylation levels of multiple kinases in the JAK-STAT and MAPK/ERK signaling pathways. These pathways were activated by the CRTC1-MAML2 fusion in H292 cells lacking EGFR mutations (Wu, He et al. 2019). Our findings provide another explanation for gefitinib response by the EGFR mutation lacking patients. However, it is unclear whether there are other somatic mutations affecting the host protein phosphorylation and therefore sensitizing the patient gefitinib response.

We have recently reported a case study of the complete response to gefitinib after the cancer surgery by a 10-year-old boy diagnosed with the low-grade EGFR-negative pulmonary mucoepidermoid carcinoma (Li, Zhang et al. 2017). The patient was cured and showed no recurrence. To investigate the tumorigenesis and gefitinib response of this patient, we performed WES analysis of the DNA samples extracted from the sputum and whole blood samples from this patient.

Methods

Sample preparation

Whole blood and sputum of one patient whose case report was published recently (Li, Zhang et al. 2017) were prepared and kept frozen until the start of this experiment. The samples were collected after approval was obtained from the Henan cancer hospital and informed consent was provided.

Exome sequencing

Genomic DNA was extracted by phenol chloroform extraction of nuclear pellets or by using a Qiagen DNeasy Blood and Tissue kit (Qiagen). Purified genomic DNAs were sheared to fragments around 100–500 base pairs and 500 ng fragmented DNAs were used for pair-end library preparation with Truseq DNA library preparation kit (Illumina). After end-repair and 3' dA overhanging, fragmented DNAs were ligated to Truseq adaptors (Illumina) and amplified for 10 cycles. Liquid phase sequence capture was performed using the NimbleGen SeqCap kit (Roche). The Truseq DNA libraries were denatured into single strand DNAs and hybridized to SeqCap oligo pools. The bound DNA fragments were eluted and PCR amplified for another 10 cycles. Fragments corresponding to 200–400 bps were purified with AMPure Xp beads and stored at -80°C until used for sequencing. Enriched libraries were sequenced on the Illumina Nextseq 500 system (ABLife Inc).

Mapping and variant analysis: Adaptors were removed from raw reads using cutadapt (version 1.7.1) at first, then reads were processed with FASTX Toolkit (version 0.0.14) for trimming low quality bases (qualities < 20) and removing low quality reads (< 70% of read length with qualities < 20). Then N-containing reads were trimmed from N base. High-quality reads longer than 16nt were aligned to the human genome (GRCh38) using BWA-MEM v 0.7.10-r789 (Li, Handsaker et al. 2009) with default parameters. The resulting alignment was sorted by coordinates and further converted to binary alignment map (BAM) format by samtools v 1.6. The rmdup module of samtools was used to remove the duplicates from the data. The Genome Analysis Tool Kit (GATK) v3.5-0-g36282e4 (McKenna, Hanna et al. 2010, DePristo, Banks et al. 2011) module RealignerTargetCreator. Indel Re-aligner and Base Re-calibrator were used to preprocess the alignments. During base quality recalibration, dbSNP variants and 1000 genome variants were used as known sites. Target-capture efficiency metrics were determined using Picard HsMetrics. The realigned and recalibrated BAM file was used as an input to GATK HaplotypeCaller using the following parameters: genotyping_mode DISCOVERY -stand_emit_conf 10 -stand_call_conf 30. Finally, raw variant calls were soft filtered using GATK Variant Filtration based on the following parameters: Low Qual (30 < Q < 50). Variants were annotated by ANNOVAR (Wang, Li et al. 2010).

Somatic mutations detection

Somatic mutations were identified using GATK Mutect2 for identification of mutations in matched tumor and normal samples. A mutation in the tumor was identified as a candidate somatic mutation only when (i) distinct paired reads contained the mutation in the tumor; (ii) the number of distinct paired reads containing a particular mutation in the tumor was at least 10% of read pairs for exome; (iii) the base depth of mutation was at least 10; and (iv) the position was covered in both the tumor and normal. Mutations arising from misplaced genome alignments, including paralogous sequences, were identified and excluded by searching the reference genome. Candidate somatic mutations were further filtered based on gene annotation to identify those occurring in protein-coding regions.

Copy Number Analysis

Copy Number Analysis from the exome data was performed using the program Control-FREEC (Boeva, Popova et al. 2012).

Functional enrichment analysis: To sort out functional categories of genes harboring somatic mutations, Gene Ontology (GO) terms and KEGG pathways were identified using KOBAS 2.0 server (Xie, Mao et al.

2011). Hypergeometric test and Benjamini-Hochberg FDR controlling procedure were used to define the enrichment of each term. Oncogenes and tumor suppressors for different cancer types were referred to the CancerMine database (<http://bionlp.bcgsc.ca/cancermine/>). All statistical analyses were carried out with two-sided tests with statistical significance level set at P value of 0.05.

Results

WES analysis of sequence nucleotide polymorphism and variations in a low grade MEC patient completely responding gefitinib

To study the somatic mutations related to the tumorigenesis and gefitinib response of MEC, we obtained whole-exome sequencing data from the frozen fresh sputum and whole blood samples from a low grade MEC patient (Li, Zhang et al. 2017). This 10-year patient was admitted to our hospital in 2012 and successfully responded to the gefitinib treatment. No relapse has been observed until now.

A total of 46,979,124 and 47,415,894 sequencing reads were generated for the sputum and blood samples, respectively. Over 97% of the targeted exon regions was covered for both samples, and 74% and 76% of targeted bases showed > 10-fold coverage for in the sputum and whole blood samples, respectively (Table S1).

Non-synonymous somatic mutations occur in tumor suppressors with a higher frequency than in oncogenes

Using the criteria described in Methods section, we identified 53 candidate somatic mutations in 50 genes in the sputum of the patient (Table S2). The mutations comprised 34 non-synonymous SNVs, 10 synonymous SNVs, 3 frameshift insertion/deletion, 3 essential splice sites, 1 stopgain, as well as 2 variations without annotations and 2 noncoding RNA mutations. Two mutations were identified in each of the following three genes, TTN (p.A4284T, p.A3465T), PGM5 (p.G215S, p.I227V) and DEAF1 (p.Y300Y, p.P299L). Five out of the six mutations were nonsynonymous mutations. A total of 12 of the 50 somatic mutation-containing genes were annotated as oncogenes and tumor-suppressor genes in the CancerMine database, which were significantly enriched among all genes (p value, 1.11×10^{-5} , hypergeometric test). Non-synonymous mutations were found in two oncogenes ADAM28 and TTN, and five tumor-suppressor genes ARRDC3, MRPL48, NPAS3, EPB41L3 and RANBP2, showing much higher frequency in tumor suppressor genes (Table 1). Additionally, nonsynonymous mutation was also observed in PRAMEF15, a member in the preferentially expressed antigen in melanoma (PRAME) family (Oberthuer, Hero et al. 2004, Epping, Wang et al. 2005, Field, Decatur et al. 2016, Hermes, Kewitz et al. 2016).

Genes harboring candidate somatic mutations were subjected to functional clustering to analyze the potential biological roles that these mutations preferentially affect. In the biological process terms of GO analysis, they only enriched in DNA-dependent transcription function (p-value greater than 0.05). In the KEGG pathway analysis, they were mostly enriched in apoptosis (RIPK1, SPTA1 and ACTG1), followed by

RNA transport (RANBP2, POM121L2 and SNORD3A). Two genes (EPB41L3 and ACTG1) were enriched in tight junction (Fig. 1A).

Non-synonymous somatic mutations were enriched in the loss and gain of Thr, Ser or Tyr

Gefitinib is an ATP analogue and well-known for its effective inhibition of the constitutively active kinase activity of the EGFR mutants (Lynch, Bell et al. 2004, Paez, Janne et al. 2004, Nyati, Morgan et al. 2006). Our recent study has shown that gefitinib inhibits the phosphorylation of multiple signaling kinases in the JAK-STAT and MAPK/ERK signaling pathways that is activated by the CRTC1-MAML2 fusion in H292 cells (Wu, He et al. 2019). We hypothesized that MEC patients might harbor phosphorylation site mutations that could sensitize some tumor-related proteins to gefitinib treatment in this patient. To test this hypothesis, we investigated the non-synonymous somatic mutations involved in the loss and gain of the protein phosphorylation amino acids Thr, Ser or Tyr. Among the 34 non-synonymous somatic mutations, we found 5 caused the gain of phosphorylation amino acids including A3636T and A4284T in TTN (Rhabdomyosarcoma Antigen MU-RMS-40.14), K135T in PRAMEF15, G215S in PGM5 (Phosphoglucosyltransferase 5), T367S in DNAH11 (Dynein Axonemal Heavy Chain 11) and P888S in RALGAPB (Ral GTPase Activating Protein Non-Catalytic Beta Subunit), and 3 caused the loss including Y76F in ARL6 (ADP Ribosylation Factor Like GTPase 6), T3219I in RANBP2 (RAN Binding Protein 2; RAN is a small GTP-binding protein of the RAS superfamily) and S880P in UBN1 (Ubinuclein 1, required for SAHF formation (Table 2). This significant high frequency of the loss (p-value, 0.023, probability test) and gain (p-value, 0.089, probability test) of the three phosphorylation amino acids in the non-synonymous somatic mutations in this studied patient might explain a part of his complete response to gefitinib.

Comparison of genes containing somatic mutations identified from this study and those from a previous study

We next compared the somatic mutations identified in this study with those identified in 18 pairs of samples from MEC patients (Kang, Tan et al. 2016). Among the 50 somatic mutation genes identified from the low-grade MEC patient in this study, 5 of them were overlapped with the reported 705 genes (Kang, Tan et al. 2016). The overlapped genes include ADAM28, DYSF, GP2, PPP2R5B, TTN, as shown in Fig. 1B. Among these overlapped somatic mutation genes, ADAM28, TTN and PPP2R5B were detected in two intermediate grade patients, and DYSF, GP2 were detected in two low grade patients in the published data.

Discussion

Whole-exome sequencing of the sputum and the matched blood of one low-grade MEC patient with a complete response to gefitinib has identified 50 genes harboring somatic mutations. About 10% of the somatic mutation-containing genes detected in our patient were also detected with somatic mutations in the previously reported MEC patients, and specifically in the low- and intermediate-grade patients (Kang, Tan et al. 2016). The consequences of these somatic mutations were mainly missense mutations, which are strongly enriched in tumor suppressors (Table 1), as well as the gain and loss of phosphorylation

amino acid Thr, Ser and Tyr (Table 2). These findings may provide new insights into the tumorigenesis of MEC and also the mechanism of gefitinib response.

Somatic mutations leading to the loss of the tumor suppressor functions are common in tumorigenesis (Gnarra, Tory et al. 1994, Sigal and Rotter 2000, Xia, Nagase et al. 2008, Wiesweg, Eberhardt et al. 2017). We found five annotated tumor suppressor protein-coding genes carry somatic mutations in our patient. EPB41L3 is well known as a tumor suppressor in a variety types of cancer including esophagus squamous cell carcinoma, lung carcinoma (Yageta, Kuramochi et al. 2002, Zeng, Liu et al. 2018). ARRDC3 and NPAS3 have been reported to suppress the progression of breast cancer and Astrocytomas (Draheim, Chen et al. 2010, Moreira, Kiehl et al. 2011, Yang, Yang et al. 2016, Xia, Han et al. 2018). RANBP2 encodes a RAN binding protein that is a small GTP-binding protein of the RAS superfamily. RANBP2 contains an unusual SUMO E3 ligase domain and directly interacts with the E2 enzyme UBC9 and strongly enhances SUMO1 transfer from UBC9 to the SUMO1 target SP100, acting as a tumor-suppressor gene in lung cancer (Dawlaty, Malureanu et al. 2008, Navarro and Bachant 2008). Knockdown of RANBP2 leads to an aberrant metaphase, mitotic arrest in G2/M phase and mitotic cell death (Hashizume, Kobayashi et al. 2013).

Oncogene mutations are also common during tumorigenesis (Mao, Hruban et al. 1994, Mills, Fishman et al. 1995, Rekhtman, Paik et al. 2012). The protein encoded by ADAM28 (ADAM Metallopeptidase Domain 28) carries self-cleavage activity and also cleaves ILGFBP3 (insulin-like growth factor binding protein-3) and connective tissue growth factor (Howard, Maciewicz et al. 2000, Mochizuki, Shimoda et al. 2004, Mochizuki, Tanaka et al. 2010). It has been reported as a potential oncogene involved in lung adenocarcinomas, non-small cell lung carcinomas, pancreatic cancer, bladder carcinoma (Mitsui, Mochizuki et al. 2006, Ohtsuka, Shiomi et al. 2006, Mochizuki and Okada 2009, Wright, Larsen et al. 2010, Tyan, Yang et al. 2011, Wei, Wen et al. 2019). Two A to T missense mutations were detected in TTN encoding the largest known protein, which plays key structural, developmental, mechanical, and regulatory roles in cardiac and skeletal muscles (Furst, Osborn et al. 1988, Vikhlyantsev and Podlubnaya 2012). An integrated analysis of the exome sequencing data from ~ 3000 tissue samples in TCGA (The Cancer Genome Atlas) database found that TTN is one the most frequent gene harboring somatic mutations in most cancer types including lung adenocarcinoma and squamous cell carcinoma, showing a potential role in cancer development or progression (Kim, Hong et al. 2013, Cheng, Yin et al. 2019). Another somatic mutation-containing S100A16 encodes a member of the S100 protein which is a ubiquitously expressed calcium-binding protein of the EF-hand superfamily which is up-regulated in tumors (Marenholz, Heizmann et al. 2004, Sturchler, Cox et al. 2006). S100A16 is a prognostic marker in multiple human cancer, including lung adenocarcinomas, breast cancer, prostate cancer, colorectal cancer, oral squamous cell carcinoma (Zhou, Pan et al. 2014, Saito, Kobayashi et al. 2015, Sapkota, Bruland et al. 2015, Zhu, Xue et al. 2016, Sun, Wang et al. 2018).

The findings that the gain and loss of phosphorylation amino acid Thr, Ser and Tyr are strongly enriched in the missense somatic mutations identified from our patient are particularly interesting. Gefitinib is an ATP analogue which effectively inhibits the constitutively active kinase activity of the EGFR mutants

(Lynch, Bell et al. 2004, Paez, Janne et al. 2004, Nyati, Morgan et al. 2006), as well as the phosphorylation of multiple signaling kinases in the JAK-STAT and MAPK/ERK signaling pathways (Wu, He et al. 2019). Given that mutation of the kinase activity is common in cancers and are the most effective class of drug targets (Sawyers 2004, Zhang, Yang et al. 2009), The accumulation of the somatic mutation altering the phosphorylation amino acids has two implications. These mutations may be selected by tumor cells because their potentials in altering the function of the host proteins. On the other side, these mutations may sensitize the host proteins to gefitinib, as does somatic mutations sensitize EGFR to gefitinib.

Conclusions

we reported 53 somatic mutations in 50 genes in one of our previously reported MEC patient. The reliability of these somatic mutation-containing genes was validated by comparison with those from a previous report. Our findings underline the possibility of the accumulated somatic mutations in the tumor suppressor genes and oncogenes might contribute to the tumorigenesis of our MEC patient. The newly identified gain and loss of the phosphorylation amino acids may represent a novel class of drug targets. Further studies are required to validate the functions of these potential functional mutations.

Abbreviations

WES:whole-exome sequencing; MEC:mucoepidermoid carcinomas;

Declarations

Ethics approval and consent to participate

Collection and use of patient samples for this study was approved by the Ethics Committee of Henan Cancer Hospital. Patient samples were obtained under a waiver of informed consent from the institution.

Consent for publication

Not applicable

Availability of data and materials

All data generated or analyzed during this study are included in this published article and its supplementary information files.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

Y.W., Y.Z. and Qiming Wang contributed to the study design. Z.H., L.W., S.Y., Y.L., B.D., Y.G. and W.W. performed the experiment, Q.W. performed data analysis, Y.W. and Yuhong Zhang prepared the manuscript. All authors read and approved the final manuscript.

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Tables

Due to technical limitations, Tables 1 & 2 are only available for download from the Supplementary Files section.

Figures

Figure 1

Results of detected somatic mutation genes in a MEC patient. (A) The top 10 representative KEGG pathways of somatic mutation genes in MEC patient. (B) Comparison of genes harboring somatic mutations of this MEC patient and published WES data.

Supplementary Files

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