Journal of Cancer Research and Clinical Oncology (2019) 145:2273–2283
https://doi.org/10.1007/s00432-019-02988-y

ORIGINAL ARTICLE – CANCER RESEARCH

REG4 is an indicator for *KRAS* mutant lung adenocarcinoma with TTF-1 low expression

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Received: 28 March 2019 / Accepted: 24 July 2019 / Published online: 19 August 2019

Abstract

Objectives Recent research has classified lung adenocarcinoma patients with *KRAS* mutation into three subtypes by co-occurring genetic events in *TP53* (KP subgroup), *STK11/LKB1* (KL subgroup) and *CDKN2A/B* inactivation plus TTF-1 low expression (KC subgroup). The aim of this study was to identify valuable biomarkers by searching the candidate molecules that contribute to lung adenocarcinoma pathogenesis, especially KC subtype.

Materials and methods We analyzed the publicly available database and identified the candidate *REG4* using the E-GEOD-31210 dataset, and then confirmed by TCGA dataset. In addition, an independent cohort of 55 clinical samples was analyzed by quantitative real-time PCR analysis. Functional studies and RNA sequencing were performed after silencing the *REG4* expression.

Results *REG4*, an important regulator of gastro-intestinal carcinogenesis, was highly expressed in *KRAS* mutant lung adenocarcinoma with low expression of TTF-1 (KC subtype). The results were validated both by gene expression analysis and immunohistochemistry study in an independent 55 clinical samples from Fudan University Shanghai Cancer Center. Further in vitro and in vivo functional assays revealed silencing *REG4* expression significantly reduces cancer cell proliferation and tumorigenesis. Moreover, RNA sequencing and GSEA analysis displayed that *REG4* knockdown might induce cell cycle arrest by regulating G2/M checkpoint and E2F targets.

Conclusion Our results indicate that *REG4* plays an important role in *KRAS*-driven lung cancer pathogenesis and is a novel biomarker of lung adenocarcinoma subtype. Future studies are required to clarify the underlying mechanisms of *REG4* in the division and proliferation of KC tumors and its potential therapeutic value.

Keywords *REG4* · *KRAS* · TTF-1 · Lung adenocarcinoma · Subtype

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Si Sun and Zhihuang Hu contributed equally to this work.

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s00432-019-02988-y) contains supplementary material, which is available to authorized users.

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Introduction

Non-small cell lung cancer (NSCLC) is one of the malignancies with the highest mortality in the world. Adenocarcinoma is the most common subtype of NSCLC (Siegel et al. 2018). The advances made in the last decade have revealed that lung adenocarcinoma (LUAC) is not a single cancer, but rather a collection of molecularly defined neoplasms with distinct biology and clinical outcomes. *KRAS* is one of the most commonly mutated oncogenes in NSCLC and is detected in 15–30% of all lung adenocarcinomas (Yu et al. 2015). The *KRAS* mutation frequency varies among different ethnic populations. In European population, *KRAS* mutation is detected in 20–30% of lung adenocarcinomas, while a relatively low frequency (15–20%) is found in Latin America and Asian populations. Zheng et al. in Fudan University Shanghai Cancer Center (FUSCC) performed the largest retrospective study of *KRAS* mutation in patients with lung adenocarcinoma in Chinese population (Zheng et al. 2016). The results showed that *KRAS* mutation occurred in 8.3% (113/1368) of the patients. Besides, the mutation is correlated with poor prognosis in the metastatic disease, and a high risk of cancer recurrence as seen in several studies. However, no effective therapies specifically targeting mutant *KRAS* have been developed to date (Ferrer et al. 2018; Pao and Girard 2011; Arrieta et al. 2015).

The development of effective treatment for *KRAS*-mutant LUACs is still a challenge because of biological heterogeneity of the *KRAS* gene and therapeutic response targeting *KRAS* or the downstream effectors of *KRAS* pathway. Lung adenocarcinomas harboring mutations in *TP53*, *STK11/LKB1*, or *CDKN2A/B* as well as *KRAS* mutation demonstrate different clinicopathological features and distinct therapeutic responses. This insight would direct the treatment strategy not only pointing at a single *KRAS* gene but a set of related genes (Ferrer et al. 2018; Tomasini et al. 2016). Skoulidis et al. directed an integrative analysis based on genomic, transcriptomic and proteomic data of *KRAS*-mutant LUAC from early-stage and chemo-refractory LUAC [The cancer Genome Atlas (TCGA)] and identified three robust subsets dominated by co-occurring genetic events in *STK11/LKB1* (the KL subgroup), *TP53* (KP subgroup) or *CDKN2A/B* inactivation plus low expression of the NKX2-1 (TTF-1) transcription factor (KC subgroup) (Skoulidis et al. 2015). The presence of commonly co-occurring mutations in tumor suppressors *TP53* and *STK11/LKB1* may represent a significant source of heterogeneity in *KRAS*-mutant tumors with different gene expression, proliferation and immune surveillance (Schabath et al. 2016). For instance, *TP53/KRAS* comutated subgroup (KP subtype) has good response to PD-1 blockade immunotherapy and may be served as a pair of potential predictive factors in guiding anti-PD-1/PD-L1 immunotherapy (Skoulidis et al. 2015; Dong et al. 2017). On the contrary, KL tumors are associated with the suppression of the tumor immune surveillance and demonstrate resistance to PD-1 immunotherapy (Schabath et al. 2016; Ji et al. 2007; Skoulidis et al. 2018). However, for KC subtype, the clinical characteristic is still unclear. Low TTF-1 expression resulting in little or no IHC staining is a defining feature of the KC subset and can be regarded as a convenient clinical biomarker facilitating their identification. Theoretically, the deletion of TTF-1 can promote the initiation and proliferation of *KRAS* mutant lung cancer, and produce a mucin phenotype similar to the gastric epithelial cell, making it evolve into a poorly differentiated and more aggressive disease (Winslow et al. 2011; Snyder et al. 2013). Clinically, the *KRAS* mutant sub-cluster membership remained a significant independent predictor of prognosis in both early and advanced or metastatic LUAD. In early stage, the prognosis of the *KRAS*-mutant LUAC subgroups was investigated from PROSPECT study using relapse-free survival (RFS) data. The results revealed that KP subtype adenocarcinomas had significant longer RFS compared to KC tumors ($P=0.0269$) (Skoulidis et al. 2015; Chitale et al. 2009). In advanced or metastatic disease, *KRAS* mutant stage IV lung adenocarcinoma with TTF-1 low expression is a highly invasive subtype with significant poor prognosis and short overall survival (7 months) (Schilsky et al. 2017).

REG4 is an important member of human regenerating protein (*REG*) family which belongs to the calcium (C-type) dependent lectin superfamily (Hartupee et al. 2001). *REG* family consists of 17 members and most of them are associated with inflammation, diabetes and cancers (Zhang et al. 2003). Increasing evidence revealed that *REG* genes were important regulators of GI carcinogenesis. The upregulation of *REG4* expression was frequently observed in many GI malignancies including colorectal, gastric and pancreatic cancers and may be considered as a new therapeutic target (Oue et al. 2005; Wang et al. 2011; Bishnupuri et al. 2006; Moon et al. 2012). Literature studies have been reported that serum levels of *REG4* were higher in pancreatic cancer patients, and knockdown of endogenous *REG4* expression in PC cell lines with small interfering RNA caused a decrease in cell viability (Eguchi et al. 2009). Overexpression of *REG4* mRNA is a potentially suitable marker of peritoneal recurrence after curative resection in gastric adenocarcinoma (Moon et al. 2012). Besides, higher levels of *REG4* expression and *REG4*-mediated genes were found to be associated with an increased resistance to apoptotic death of human colorectal cancer cells (Bishnupuri et al. 2006). These studies suggested an association of *REG4* with poor patient outcomes in human GI cancers.

In this study, we performed bioinformatics analysis from the publicly available E-GEOD-31210 microarray and The Cancer Genome Atlas (TCGA) database. For the first time, we found *REG4*, an important regulator of gastro-intestinal (GI) carcinogenesis, was highly expressed in *KRAS*-mutated lung adenocarcinoma. Further study of independent 55 clinical samples from Fudan University Shanghai Cancer Center (FUSCC cohort) and TCGA cohort showed that *REG4* was significantly elevated in KC cluster than KP and KL cluster. The effects of silencing *REG4* expression on tumor progression were investigated both in vitro and in vivo. Targeting of *REG4* gene expression by siRNA significantly inhibited the proliferation of human lung adenocarcinoma cell lines and experimental animal models. Our results indicate that *REG4* plays an important role in *KRAS*-driven lung cancer pathogenesis and is a novel biomarker of lung adenocarcinoma subtype.

Materials and methods

Bioinformatics analysis from public microarray profile

We selected a published Affymetrix microarray dataset E-GEOD-31210 (www.ebi.ac.uk/arrayexpress) for bioinformatics analysis, which included gene expression profiles from 226 lung adenocarcinomas and 20 adjacent normal tissue samples. There are 127 samples with EGFR mutations, 11 samples with EML4-ALK fusions, 20 samples with *KRAS* mutations, and 68 triple-negative cases samples. R software and the Bioconductor package were utilized to analyze the downloaded Raw data CEL files. After preprocessing with RMA (Robust Multi-chip Average) background correction, quantile normalization, and median polish summarization, gene expression values were ready for downstream analysis. With the aim to pick up genes that expressed differentially, 8658 Probesets with mean signal intensity in those patients below 20 was kept for further analysis. Probeset 223447_at exhibited the highest fold changes when comparing patients with *KRAS* mutation and patients with ALK rearrangement or EGFR mutations.

TCGA dataset analysis

The Cancer Genome Atlas Lung Adenocarcinoma (TCGA-LUAD) on RNA expression (Level 3) of lung adenocarcinoma patients in terms of RNA-seq by Expectation–Maximization was downloaded from the Cancer Genomics Brower of the University of California, Santa Cruz (UCSC) (https://genomecancer.ucsc.edu/). Totally, 230 primary lung adenocarcinoma samples from patients with detailed expression data were chosen from the updated TCGA database according to the parameters mentioned. Detailed demographics of these patients were characterized by the TCGA consortium.

Cancer cell lines and clinical samples

We selected two cell lines, DV90 and NCI-H727 which has *KRAS* mutation and *REG4* high expression. ATCC cells were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum and 2 mmol-glutamine, in 6-cm dishes. We analyzed 55 NSCLC tumor samples with *KRAS* mutation from FUSCC cohort. All the patients were at early-stage or locally advanced diseases and undergone surgical resection. All participants signed the informed consent under the approved study from the Ethics Committee. The study was performed according to the ethical standards of the Responsible Committee on Human Experimentation of Fudan University Shanghai Cancer Center.

Real-time PCR detection

RNA was isolated from frozen tissue samples levigated in TRIZOL solution, using a RNA easy Plus Mini Kit from Qiagen. cDNA was synthesized using QuantiTect Reverse Transcription kit (Qiagen). Quantitative real-time PCR was performed on an ABI 7900HT instrument using the QuantiFast SYBR Green PCR kit (Qiagen). Two-step cycling was performed for 40 cycles, with 10 s at 95 °C and 30 s at 60 °C, after initial activation for 5 min at 95 °C, followed by melting curve analysis. The expression of the *HPRT1* and *ESD* genes in lung cancer tissue was selected for normalization.

Immunohistochemistry (IHC)

All specimens used in this study were 4-µm-thick sections of formalin-fixed, paraffin-embedded tissue obtained at surgery. The anti-TTF1 antibody TTF1 (8G7G3/1, DAKO, Glostrup, Denmark) and anti-REG4 antibody (mouse monoclonal antibody, Abcam, UK) was used at a dilution of 1/100 and 1/200, respectively. Immunohistochemical staining was carried out using a standard streptavidin–biotin-based method. Slides were blindly reviewed by two pathologists. For each tumor, neoplastic cells were assessed as positive or negative for TTF1 staining. For REG4 staining, the number of immunopositive cells was semiquantitatively estimated: negative, < 30% of the tumor cells staining positive; positive, 30–100% of tumor cells staining positive.

RNA interference of *REG4* and the cell proliferation assay

The small interfering RNAs (siRNAs) against *REG4* as well as a scramble normal control siRNA (NC) were synthesized by Ribobio (Guangzhou, China). DV90 and NCI-H727 cells were transfected with the siRNAs using Lipofectamine RNAiMax, and the expressions of *REG4* were assessed after 48 h of incubation. To measure the effect of *REG4* siRNA on the tumor cell proliferation, CCK-8 (Dojindo, Japan) assays were performed according to the protocol of the manufacturer.

In vivo xenograft models

DV90 cells stably expressing lentiviral short hairpin RNA against *REG4* (sh-REG4) or control were harvested and suspended in RPMI-1640. Each mouse (male BALB/c-nu/nu, 6-week-old) was injected subcutaneously in the lower back with 0.2 ml of RPMI-1640 containing 2×10^6 cells. The mice were killed after 7 weeks and xenograft tumors were excised and weighed. All animal studies were manipulated using protocols approved by the Shanghai Medical Experimental Animal Care Commission.

RNA-seq and Gene Set Enrichment Analysis (GSEA)

RNA from DV-90 cells with or without *REG4* knockdown was extracted and purified for quantification. RNA-seq library was prepared using a TruSeq RNA library preparation kit in accordance with the manufacturer's instructions. To identify the enriched transcriptomic signatures with the expression of *REG4* on *KRAS*-mutated cell line (DV90), GSEA was adopted and the java GSEA Desktop Application was downloaded from http://software.broadinstitute.org/gsea/msigdb/index.jsp. The genes identified to be on the leading edge of the enrichment profile were subject to pathway analysis. The normalized enrichment score (NES) is the primary statistic for examining gene set enrichment results. The nominal *P* value estimates the statistical significance of the enrichment score and $P \leq 0.05$ is measured to find enrichment with statistical significance in a gene set.

Western blot analysis

For western blot analysis, cells from the control and experimental groups were lysed using the RIPA buffer (Sigma) containing phosphatase and protease inhibitors according to the manufacturer's directions. Protein lysates were subjected to SDS-PAGE electrophoresis and transferred to PVDF membrane (Millipore, Bedford, MA, USA). Antibodies of β-actin, cyclin D1 and p21 were purchased from Proteintech Group, Inc (Rosemont, IL).

Statistical analyses

Statistical analyses were conducted using GraphPad Prism (version 7.0, La Jolla, CA) and SPSS version 22.0. Scatter dot plot and box and whisker plots indicate median and 95% confidence interval (CI). Statistical tests were used to analyze the clinical and genomic data, including the Chi-square and Fisher's exact. All reported *P* values are two-tailed and $P \leq 0.05$ is considered statistically significant for all analysis.

Results

The expression of *REG4* is associated with *KRAS* mutation

By comparing the expression profiles of 226 lung adenocarcinoma samples from the E-GEOD-31210 dataset, probe set 238984_at corresponded to *REG4* was found to be the most differentially expressed gene. *REG4* expression significantly elevated in *KRAS*-mutated LUAC samples only, but rarely showed expression in other sample types (*EGFR* mutation; *ALK* fusion; Negative for *ALK* fusion, *EGFR* mutation and *KRAS* mutation; adjacent normal tissue) (Fig. 1). The strong correlation between *REG4* expression and *KRAS* mutation was further confirmed in the TCGA dataset. It revealed that *REG4* upregulation was specifically related to *KRAS*-mutated lung adenocarcinoma and accounted for approximately 8% of this type (Fig. 2a). *REG4* upregulation was enriched in *KRAS* mutant group when compared to *KRAS* wild-type group ($P < 0.0001$) (Fig. 2b).

REG4 increased significantly in *KRAS* mutant combined with TTF-1 low expression subtype (KC subtype)

To further clarify the relationship between *REG4* and *KRAS* mutant lung adenocarcinoma, we analyzed 57 cases of *KRAS* mutation of lung adenocarcinoma in the TCGA database, of which 20 were combined with *TP53* gene mutation (KP subtype), 23 cases with *LKB1* gene mutation (KL subtype) and 14 cases with TTF-1 low expression (KC subtype). The expression level of *REG4* gene in KC subgroup was significantly higher than that in group of KP and KL, $P < 0.0001$ (Fig. 3). Correspondingly, 55 cases of *KRAS* mutant lung adenocarcinoma samples were collected from the FUSCC cohort. *REG4* gene was detected by real-time PCR and the relationship between *REG4* and *KRAS* subtype was analyzed. Among the 55 cases, 11 were KC subgroup with low expression of TTF-1. The remaining 44 cases were KP or KL subgroup with TTF-1

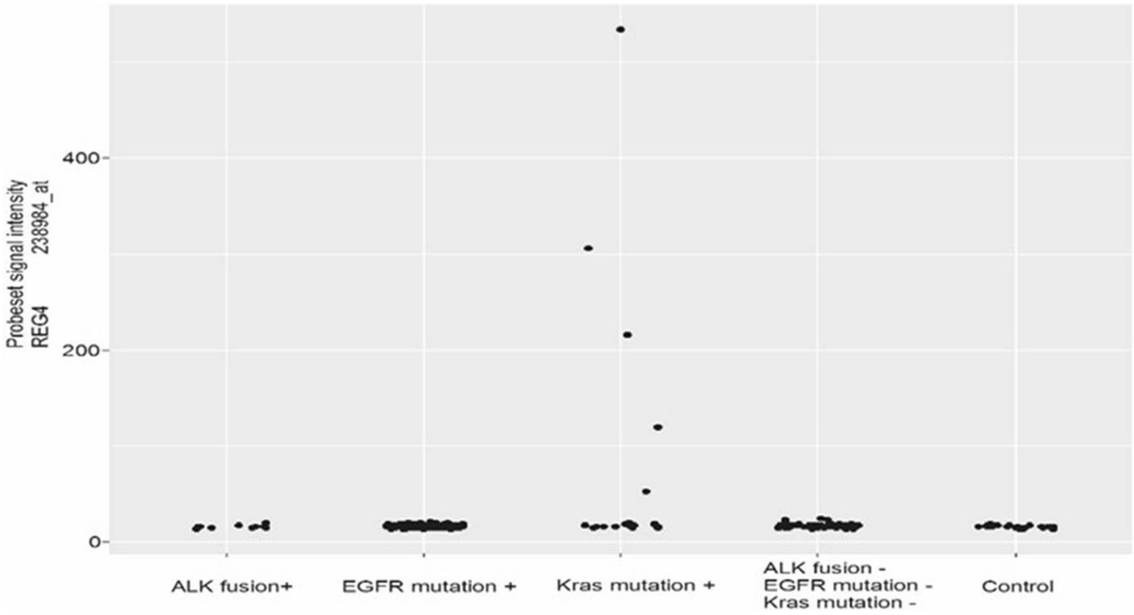

Fig. 1 Distinctive *REG4* expressions between LUAC subtypes. From left to right, 11 cases were *ALK* fusion gene positive, 127 cases were *EGFR* mutation, 20 cases were *KRAS* mutation, 68 cases were *ALK/EGFR/KRAS* negative, and 20 cases were adjacent normal tissue. The results showed that *REG4* was highly expressed only in some *KRAS* mutant lung adenocarcinomas, and almost all of them were negative in wild-type *KRAS* lung adenocarcinomas

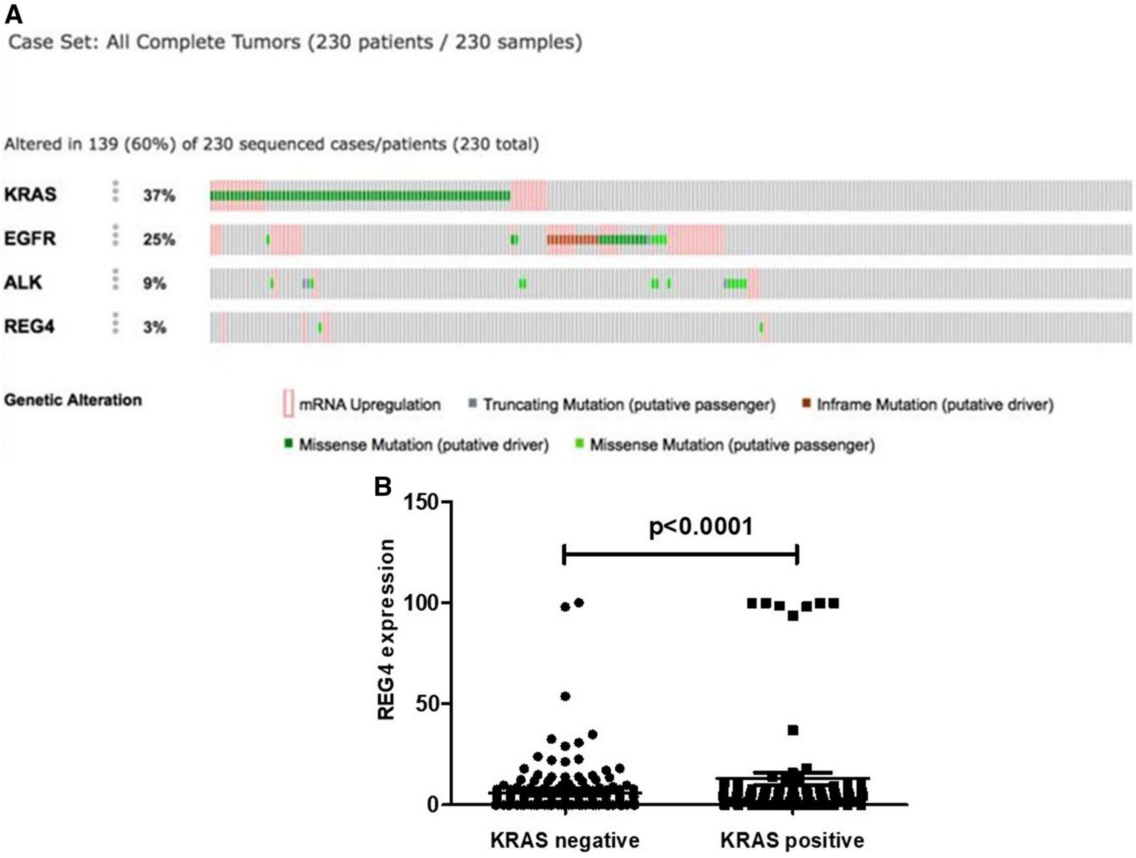

Fig. 2 Co-occurring genetic events in TCGA dataset. **a** *REG4* expression almost exists in *KRAS* mutant lung adenocarcinoma (red indicates elevated expression of mRNA). **b** Comparison of *REG4* expression in *KRAS* mutant and wild-type group

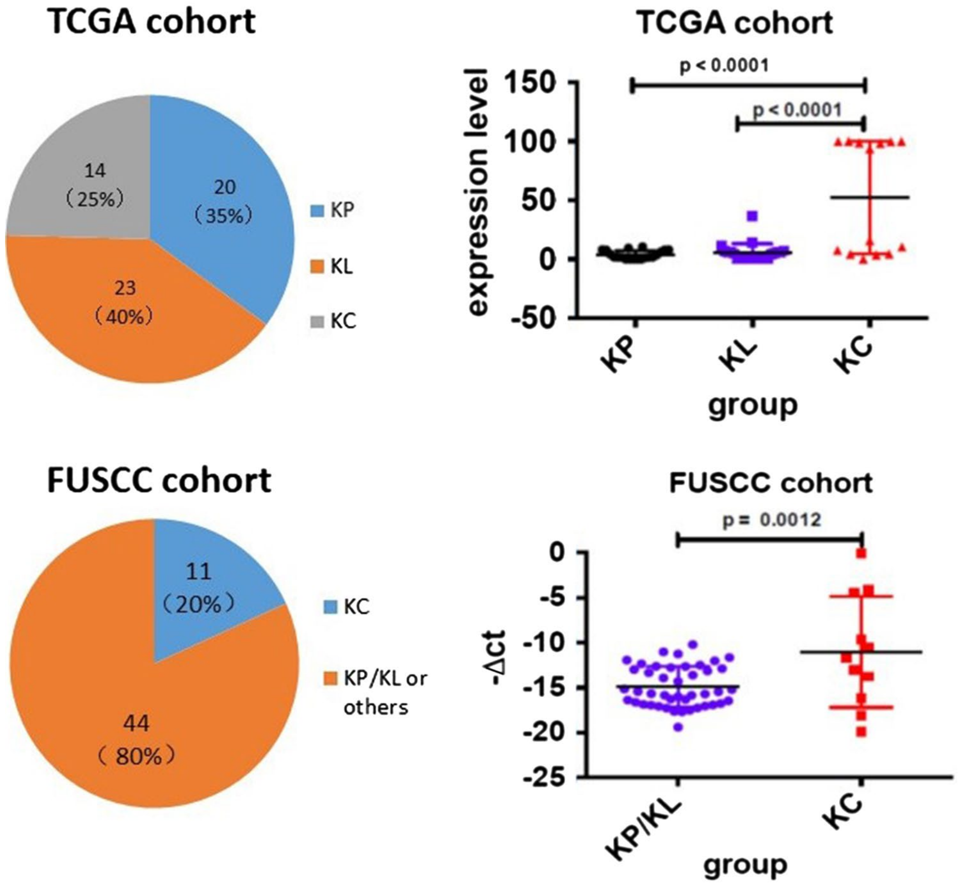

Fig. 3 Distribution of KP/KL/KC subtypes in TCGA database and FUSCC cohort and their relationship with *REG4* expression

expression. Analysis showed that the expression of *REG4* was also significantly higher in KC subgroup, $P < 0.01$ (Fig. 3).

Besides, we performed IHC staining in 40 cases of clinical specimen in FUSCC cohort to further validate the REG4 protein expression in TTF-1 suppressed subgroup (KC subgroup) and TTF-1-positive subgroup (KP/KL subgroup). The results showed that REG4 expression was also significantly higher in KC group compared with KP/KL group ($P = 0.0095$, Fig. 4a). The representative imagines are displayed in Fig. 4b and revealed that REG4 expression was confined to TTF-1-negative group and on the contrary, almost negative in TTF-1-positive group.

REG4 contributes to proliferation of lung cancer cells

To investigate the role of *REG4* in tumor proliferation, we chose DV90 and NCI-H727 cell lines with relatively high *REG4* expression. Two small interference RNA fragments (si-*REG4*-1, si-*REG4*-2) were designed for inhibiting the *REG4* gene, and the silencing effect was validated using real-time PCR (Fig. 5a). The proliferation, apoptosis rate, migration and invasion ability of tumor cells were analyzed. The results of CCK8 test confirmed that the proliferation of DV90 and NCI-H727 cells was significantly inhibited after interfering with *REG4* gene ($P < 0.05$) (Fig. 5b, c), but there was no significant change in cell apoptosis and migration and invasion, suggesting that *REG4* could promote the proliferation of lung cancer cells. We further established stable *REG4* knockdown cells using lentiviral short-hairpin RNA. The inhibition of *REG4* expression dramatically inhibited the tumorigenic ability and reduced the tumor volume of the DV90 cells in xenograft mouse models (Fig. 5d, e).

Identification of *REG4*-regulated genes in siRNA-treated DV90 cells

To investigate if the target genes are involved in si-*REG4*-RNA-treated DV-90 cells, we performed RNA-seq to profile the transcriptome changes after knocking down *REG4* in DV90 cells (Accession number GSE132011). In total, 73 genes with significantly differential expression accompanied by *REG4* knockdown were identified ($P < 0.05$). Among them, 50 genes were upregulated and 23 were downregulated (Supplementary Table 1). Furthermore, GSEA enrichment analysis for the differentially expressed genes (DEGs) was conducted and the top eleven significantly enriched GSEA

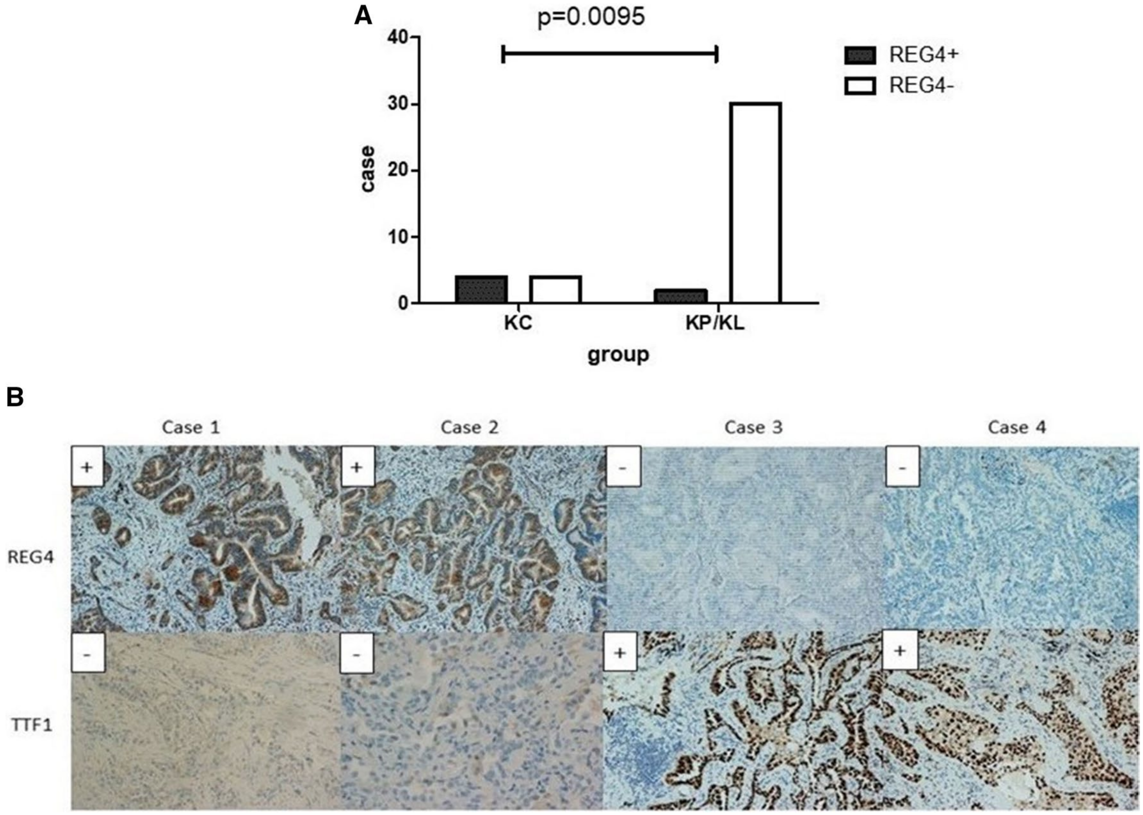

Fig. 4 **a** IHC staining revealed REG4 protein expression was higher in KC group. **b** Representative imagines of REG4 expression in TTF1− (KC) and TTF1+ (KP/KL) groups

pathways are demonstrated in Fig. 6b, including E2F signaling ($P<0.001$, FDR $=0.09$), G2 M signaling ($P<0.001$, FDR $=0.05$), MTORC1 signaling ($P=0.01$, FDR $=0.15$) and P53 pathway ($P=0.009$, FDR $=0.132$). The cell cycle-related genes such as cyclin D1/2 (CCDN1/2) were decreased and p21/p15 were elevated (Fig. 6c). The expression of cyclin D1 and p21 was validated through western blot in DV90 cells following REG4 knockdown (Fig. 6d).

Prognosis of KC subtype and the role of *REG4* expression

To further explore the prognosis of KC subtype and the role of *REG4* expression, we also analyzed the OS and RFS data from FUSCC dataset. Our results revealed that KC tumors had significantly shorter OS ($P<0.001$, log-rank test) (Fig. 7a) and a trend of shorter RFS ($P=0.085$, log-rank test) (Fig. 7b) compared to KP and KL LUACs combined. However, higher expression of *REG4* did not predict quick relapse in either KC cluster alone or *KRAS* mutant tumors in total (data not shown). As the number of KC tumors is limited with only 11 cases in our cohort, a larger, prospectively collected dataset was required for the evaluation of the role of *REG4* in predicting the prognosis of KC tumors.

Discussion

Recently studies demonstrated that the lung adenocarcinoma harboring *KRAS* mutation is not an isolated disease but contains different kinds of genetic heterogeneity. This is approved by the distinct clinicopathological features and different therapeutic responses when *KRAS* mutation co-occurring with other gene mutation or immunohistochemical phenotype (Schabath et al. 2016). Skoulidis et al. were the first to perform an integrative analysis of genomic, transcriptomic and proteomic data from LUAC and identified three robust subsets of *KRAS*-mutant LUAC (Skoulidis et al. 2015). This insight might direct distinct treatment strategies not only pointing at a single *KRAS* gene, but considering the presence of the major co-occurring genes.

In this study, we focus on KC subtype, a rarely investigated cluster compared with KP and KL subtypes. The low expression of TTF-1 (NKX2-1) is a defining feature of this subset. The transcription factor TTF-1 (NKX2-1) is a master regulator of pulmonary differentiation which routinely assessed in diagnostic histopathology of suspected lung cancers with the combination of biomarkers, such as napsin A. TTF-1 is detected in 80–85% of LUACs (Ye et al. 2011; Ikeda et al. 2015), but is almost uniformly suppressed in

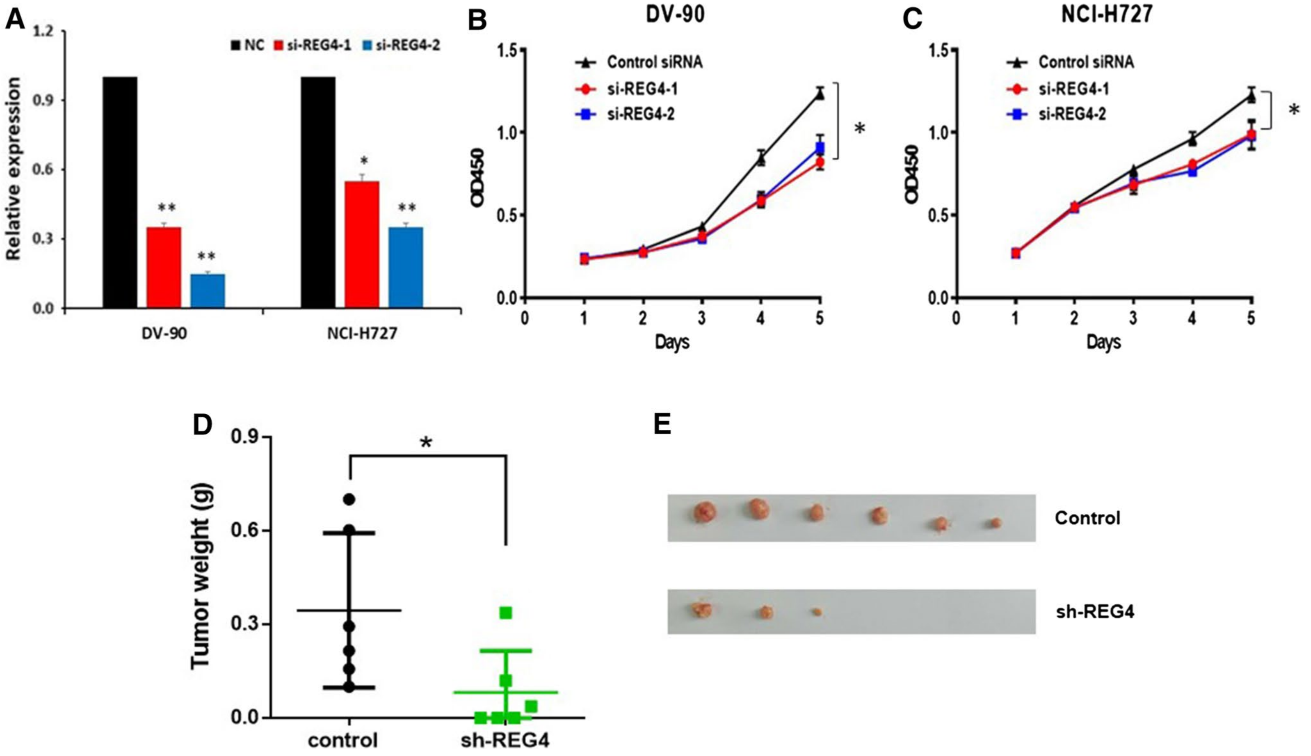

Fig. 5 REG4 contributes to proliferation of KRAS mutant cancer. **a** Two small interference RNA fragments (si-REG4-1, si-REG4-2) were designed for inhibiting the *REG4* gene, and the silencing effect was validated using real-time PCR. **b**, **c** Silencing *REG4* expression decreased proliferation of DV90 (**b**) and NCI-H727 (**c**) cells reflected by CCK-8 proliferation assay. **d**, **e** Silencing *REG4* expression reduced tumorigenesis of DV90 cells in xenograft mouse models, control ($n=6$) and sh-*REG4* ($n=6$, only three mice with tumor formation)

KC subtype tumors and may represent a clinical biomarker to facilitate their identification. Although characteristic co-occurring genomic events could not be found in many tumors in the KC cluster, the recent research indicated that *CDKN2A/B* bi-allelic loss was enriched and may impact its differentiation (Skoulidis et al. 2015). The preclinical studies demonstrated that the simultaneous of *KRAS* activation and NKX2-1(TTF-1) deletion yielded invasive adenocarcinomas in the peripheral lung which exhibited a dramatically altered differentiation state compared to NKX2-1 (TTF-1)-positive tumors and drives a distinct, GI like differentiation program (Snyder et al. 2013). In agreement with the biological characteristics, the prognosis of KC cluster is unsatisfied. In patients with early-stage lung adenocarcinomas following surgical resection, RFS data from PROSPECT were explored and revealed that KC tumors had significantly shorter RFS compared to KP and KL LUACs (Skoulidis et al. 2015). Our results also revealed shorter OS and a trend of shorter RFS in KC patients after surgery. In patients with advanced or metastatic disease, *KRAS* and TTF-1 are also predictive and/or prognostic markers. KC subtype was associated with much shorter OS compared with non-KC tumors (*KRAS* mutation and TTF-1 positive) (7 months vs 13 months, $P<0.0001$) (Schilsky et al. 2017). Thus, further research is needed to examine the physiologic mechanism underlying this subtype and explore the opportunity to precisely identify therapies for patients with KC tumors.

For the first time, we found *REG4* was highly expressed in *KRAS*-mutated lung adenocarcinoma, especially in *KRAS*-mutated lung adenocarcinoma with low expression of TTF-1 (KC subtype). REG4 was previously reported to be an important regulator of GI carcinogenesis, which associated with the initiation and progression of cancer and considered to be a new therapeutic target. The recent study reported the production of an engineered immunoglobin, a single-chain antibody (scFv–Reg4), to specifically bind REG4 and significantly inhibited gastric cancer cell proliferation (Zhang et al. 2019). As exhibited in our results, *REG4* upregulation is a unique genetic and immunohistochemical phenotype in KC tumors, which was not detected in the KL and KP subgroups. In agreement with the role for TTF-1/NKX2-1 deletion in driving a latent gastric differentiation program in the context of mutant *KRAS* (Winslow et al. 2011; Snyder et al. 2013), we suppose that *REG4* might play an important role in initiating KC tumor carcinogenesis. Besides, the inhibition of *REG4* leads to the suppression of KC cancer

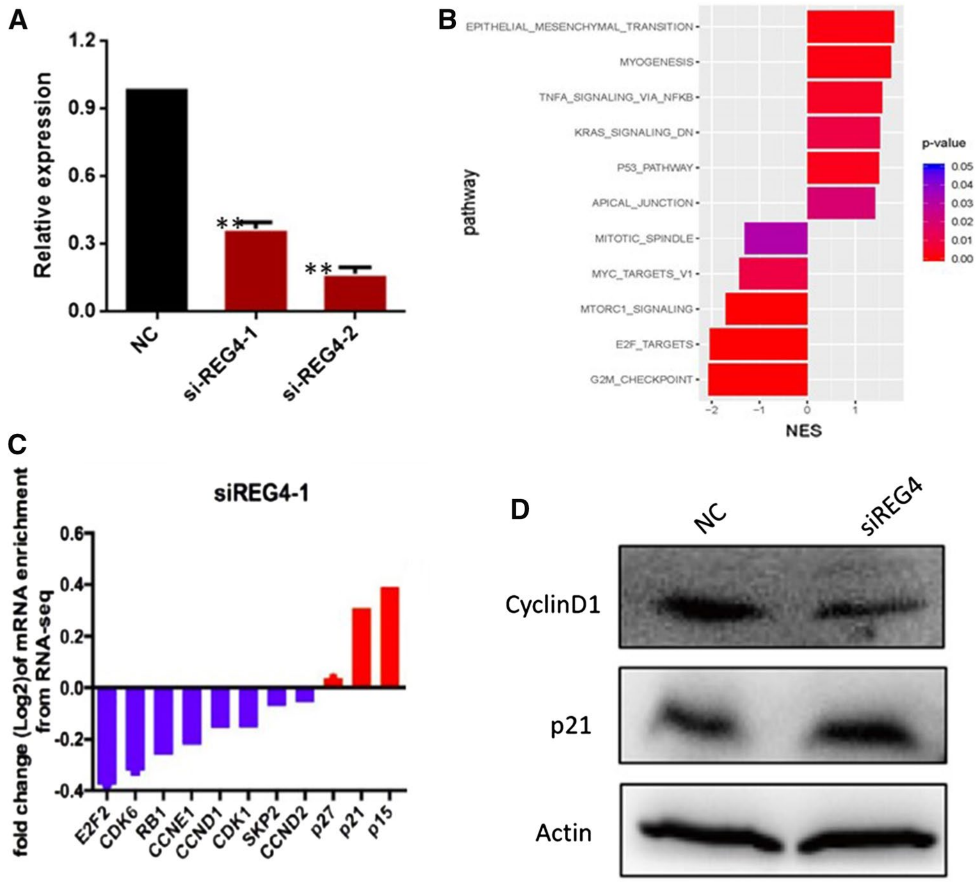

Fig. 6 GSEA enrichment analysis predicting four significant pathways regulated by *REG4* expression. **a** Two siRNA against *REG4* were transfected in DV90 cells for 48 h to examine the decrease of *REG4* mRNA. **b** The top eleven significantly enriched GSEA pathways with the decrease of *REG4*. **c** The cell cycle-related genes such as cyclin D1/2 (CCDN1/2) were decreased and p21/p15 were elevated. **d** The expression of cyclin D1 and p21 was validated through western blot in DV90 cells following REG4 knockdown

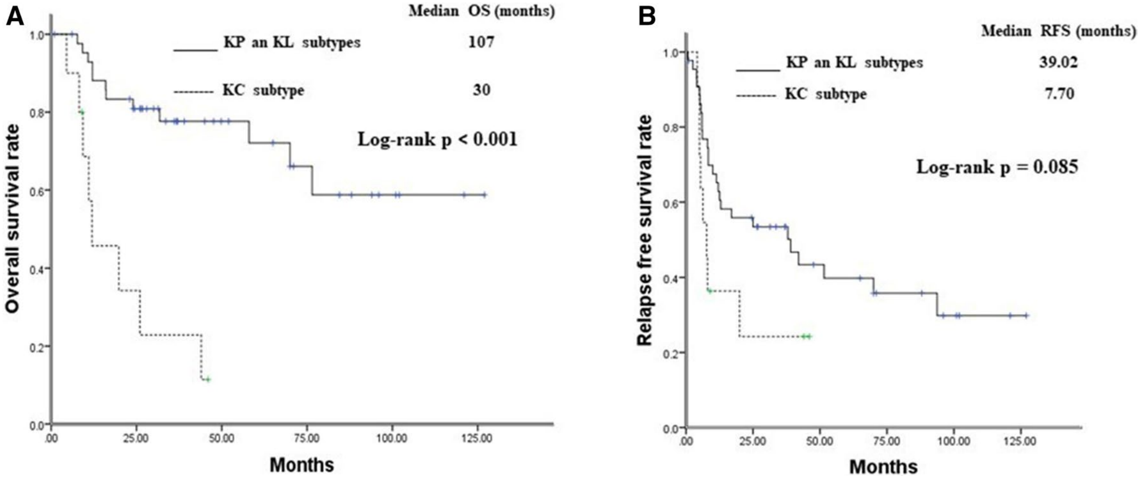

Fig. 7 Kaplan–Meier analysis of OS (**a**) and RFS (**b**) following surgical resection of 55 *KRAS*-mutant LUACs from FUSCC cohort

cell growth both in vitro and in vivo, suggesting a potential therapeutic benefit in neutralizing *REG4* activity in *REG4*-positive patient populations.

Furthermore, the function and the potential molecular mechanism of *REG4* in KC tumors were also investigated in vitro. *REG4* is regarded as an important regulator of cell cycle. It was demonstrated that knockdown of *REG4* expression resulted in the upregulation of cell cycle inhibitors *p21* and *p27* and induces cell growth (Vanderlaag et al. 2012). As reported in human colorectal cancer, *REG4*-induced mitogenesis is related to Akt–GSK3β–β-catenin–TCF-4 signaling pathway. *REG4* upregulation significantly increased the expression of cell cycle-related genes *cyclin D1* and *D3*, and was associated with cyclin-dependent kinases *CDK4* and *CDK6* (Bishnupuri et al. 2014). In prostate cancer cells, knockdown of *REG4* also resulted in the induction of *p21* expression, which negatively regulates *cyclin D1* and blocks the G1/S transition (Liu et al. 2013). In our study, we explored the function of *REG4* in KC cell lines (DV90 and NCI-H727 cell lines). Silencing *REG4* significantly inhibited the proliferation of *KRAS*-mutated DV-90 and NCI-H727 cells, indicating that *REG4* was an important regulator of KC subtype. RNA sequencing and GSEA analysis revealed that *REG4* downregulation might induce the cell cycle arrest by regulating G2/M checkpoint and E2F targets, including the decrease of *cyclin D1/2* and increase of *p21/p16* expression. The result was also verified in protein level by western blot analysis. Considering *TTF-1(NKX2-1)* may contribute to lung adenocarcinoma proliferation via *cyclin D1* expression and depletion of *NKX2-1* by siRNAs strongly suppressed the cyclin D1 protein (Harada et al. 2017), we predict that *REG4* plays an important role in triggering key events in cell cycle progression of KC tumors. Besides, as KC subtype displayed high basal level of wild-type *TP53*-regulated transcripts (Skoulidis et al. 2015), our finding is consistent with wild-type *TP53* status and is further supported by high rates of *TP53* increase with the inhibition of *REG4*.

Taken together, the results of this study highlighted an insight that *REG4* was highly expressed in *KRAS* mutation with TTF-1 showing low expression in lung adenocarcinoma. Functionally, *REG4* is responsible for cancer cell proliferation and tumorigenesis. Mechanistically, we had discovered that *REG4* downregulation induced the cell cycle arrest by regulating the E2F pathway and blocks the G2/S transition. These studies provided evidence that *REG4* may play an important role in *KRAS*-driven lung cancer pathogenesis. Further studies are warranted to evaluate the underlying mechanism of *REG4* in the division and proliferation of KC tumors and the potential clinical value.

Funding This work was supported by a grant from the National Natural Science Foundation for Young Scientists [Grant number 81501965, Dr. Hui Yu].

Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest.

Ethical approval The study was approved by the Ethics Committee of Fudan University Shanghai Cancer Center and carried out in accordance with the 1964 Helsinki declaration and its later amendments. All patients provided written informed consent before any study-related procedure. All animal studies were manipulated using protocols approved by the Shanghai Medical Experimental Animal Care Commission.

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