The transcription factor forkhead box P3 (FOXP3) is a biomarker for regulatory T cells and can also be expressed in cancer cells, but its function in cancer appears to be divergent. The role of hepatocyte-expressed FOXP3 in hepatocellular carcinoma (HCC) is unknown. Here, we collected tumor samples and clinical information from 115 HCC patients and used five human cancer cell lines. We examined FOXP3 mRNA sequences for mutations, used a luciferase assay to assess promoter activities of FOXP3’s target genes, and employed mouse tumor models to confirm in vitro results. We detected mutations in the FKH domain of FOXP3 in 33% of the HCC tumor tissues, but in none of the adjacent nontumor tissues. None of the mutations occurred at high frequency, indicating that they occurred randomly. Notably, the mutations were not detected in the corresponding regions of FOXP3 genomic DNA, and many of them resulted in amino acid substitutions in the FKH region, altering FOXP3’s subcellular localization. FOXP3 delocalization from the nucleus to the cytoplasm caused loss of transcriptional regulation of its target genes, inactivated its tumor-inhibitory capability, and changed cellular responses to histone deacetylase (HDAC) inhibitors. More complex FKH mutations appeared to be associated with worse progression in HCC patients. We conclude that mutations in the FKH domain of FOXP3 mRNA frequently occur in HCC and that these mutations are caused by errors in transcription and are not derived from genomic DNA mutations. Our results suggest that transcriptional mutagenesis of FOXP3 plays a role in HCC.

Hepatocellular carcinoma (HCC) is one of the most common malignancies worldwide and its tumorigenesis is a multi-step process. It has been well-revealed that transcription factors, which are frequently mutated, contribute to the development and progression of HCC. FOXP3 is a member of the forkhead box (FOX) transcription factor family that plays complex and important roles in cell growth, proliferation, and differentiation. Although this transcription factor is mainly studied in regulatory T cells, increasing evidence has indicated that its expression in tumor cells is associated with the progression of various cancers. Moreover, functional studies have demonstrated that FOXP3 overexpression may suppress the proliferation of cancer cells, indicating that FOXP3 may play inhibitory roles at least in certain types of cancers.

Similar to some actively studied transcription factors, such as p53, FOXP3 is mutated in some cancers, including breast cancer and prostate cancer. Notably, some of the mutations are detected in forkhead (FKH) domain, which is critical for transcriptional activities of FOX proteins. However, it remains unclear whether and how FOXP3 mutations may influence the progression of cancers. In this report, we have demonstrated that mutations in the FKH domain of FOXP3 were frequently detected in HCC tumor tissues but not in nontumorous tissues. Interestingly, the mutations were only identified in FOXP3 transcripts but not at the genomic DNA level. Most of the point mutations resulted in amino acid substitutions, which could subsequently cause changes of FOXP3 subcellular localization, and attenuate the transcriptional activity and the tumor-inhibitory capability of FOXP3. Our results have suggested for the first time that the transcriptional mutagenesis of FOXP3 may play remarkable roles in HCC growth and/or development.

The abbreviations used are: HCC, hepatocellular carcinoma; FKH, forkhead; HDAC, histone deacetylase; TSA, trichostatin A; VPA, valproic acid; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.
FKH mutations were frequently detected in FOXP3 transcripts of HCC tissue samples. In total, 115 pairs of tumorous and their corresponding nontumorous tissues were screened and FKH mutations were detected in 38 tumors, about 33% of total samples, but in none of the nontumorous tissues. All the point mutations were shown in a heterozygous pattern, suggesting the detection of mutant and WT alleles at the same position (Fig. 1, A and B). However, in some cases, mutant alleles demonstrated a lower proportion (HCC1_T) whereas in some other cases mutations showed a higher proportion (HCC2_T) compared with that of WT signals. Among the point mutations, the transition rate (G/A or C/T) was much higher than that of the transversion (A/T or C/G). Some mutations caused amino acid changes (nonsynonymous) whereas some did not (synonymous). However, no prevalent point mutations were detected at high frequencies in the samples, indicating that the mutagenesis occurred randomly.

Single point mutation in FKH region was observed in 21 of the 38 tumors. In the other 17 samples, at least 2 obvious point mutations were detected in FKH region and in some cases messy signals indicating the mixture of different sequences were observed in certain regions within the FKH domain of FOXP3 mRNA in tumors but in none of the nontumorous tissues (Fig. 1B). To examine the details of the mixed sequences, PCR products of the FKH domain were cloned and sequenced. Results showed in the messy region various mutations existed along with the WT sequence. Some mutations caused amino acid changes (AAG>ATG, GAG>GGG etc.) and some deletions led to ORF frameshift (TTT>T-T) (Fig. 1B). The mutations were not observed at genomic DNA levels (Fig. 1B).

To rule out the possibility that the FOXP3 mutants were amplified from tumor-infiltrating lymphocytes instead of can-

**Figure 1.** Mutations were detected in FKH domains of FOXP3 transcripts in HCC. A, representative sequencing chromatograms of point mutations. B, representative sequencing chromatograms of the complicated mutation status. Upper left panel, FKH sequences in mRNA. Upper right panel, sequences of the corresponding regions in genomic DNA. Lower panel, sequences of individual TA clones which were generated to examine individual sequences in multiple-mutation bearing PCR products. C, representative immunohistochemical results of FOXP3-positive lymphocyte distribution in tumors and corresponding nontumorous tissues. Black arrow, FOXP3-positive lymphocytes.
cer cells, immunohistochemistry was performed to examine the distribution of lymphocytes in liver tissue samples. The results clearly demonstrated the presence of FOXP3-positive lymphocytes in both tumorous and nontumorous tissues (Fig. 1C). Because no mutations were found in nontumorous tissues, the finding indicated that the detected FOXP3 mutations were derived from the cancer cells but not from the infiltrating lymphocytes.

To assess the role of FKH mutations in the prognosis of the tumor, we analyzed the relationship of FKH mutation status with clinical pathological features. The result showed that FKH mutation in tumors had no significant association with survival time. However, poorer prognosis was observed in the 17 patients with two or more point mutations in FKH compared with that of the 77 patients without FKH mutations. 10 of the 17 patients died within 12 months after surgery and the 1-year death rate was significantly higher than that of the patients without FKH mutations (Fig. 2A). Moreover, the survival time of the 17 patients was significantly shorter than that of the patients without FKH mutations. In addition, several clinical parameters were significantly different between the patients with FKH mutations and those without, such as severe cirrhosis (grade C), vascular invasion, as well as AJCC grading (Table S1).

To investigate the possible cause of FKH transcript mutations, we examined POLR2I that encodes enzyme RPB9, a subunit of RNA polymerase II. RPB9 controls the transcription fidelity (11), and its defect can significantly increase the rate of the error transcription by 2- to 3-fold (11, 12), which likely contributes to the FKH mutations found in this study. We found that the expression of POLR2I was reduced in most of the 115 HCC tumors when compared with that of the corresponding nontumorous tissues, but the difference was not significant (Fig. 2B, left panel). However, the significant down-regulation of POLR2I expression occurred in all 38 HCC tumors with FKH mutations detected (Fig. 2B, right panel). It was reported that HDAC inhibitors such as SAHA regulated the transcriptional activity by repressing RNA polymerase II elongation complex (13). We therefore treated cells with SAHA and found that the expression of POLR2I was suppressed in HCC Hep3B and Huh7 cells but not in other cells (Fig. 2C). The results further implicated the close relationship between the insufficient expression of POLR2I and the transcription errors.

Figure 2. Down-regulation of POLR2I gene expression correlates with FKH transcription errors. A, complicated FKH mutation status is associated with patient survival after surgery. *, p < 0.05 compared with that of No Mutation group. B, gene expression of POLR2I was suppressed in HCC. POLR2I mRNA levels were normalized with that of β-actin mRNA. The data are expressed as -fold changes relative to the expression level in nontumorous tissue of respective HCC patient. Each column represents the mean ± S.D. of data obtained from all 115 HCCs of this study (left panel) or the 38 HCCs in which FKH mutations were observed (right panel). C, HDAC inhibitor SAHA down-regulated POLR2I expression and induced FOXP3 transcription error in liver cancer cell Hep3B and Huh7. Left, representative sequencing chromatograms showing point mutations in FKH domain. Right, gene expression of POLR2I.
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Nonsynonymous FKH mutations changed FOXP3 subcellular localization

Mutant FKH-bearing pMutFOXP3Egfp plasmids were constructed by replacing the original WT FKH domain in pFOXP3EGFP with the mutant FKH domains amplified from HCC samples (Fig. 1). The plasmids were then transfected into HCC PLC/PRF/5 cells and the subcellular distribution of EGFP fusion protein was observed. Nonsynonymous FKH mutations caused the change of FOXP3 localization which was originally in the nucleus exclusively. For example, mutations K393M/E400G, L389P, and L344P resulted in the exclusive cytoplasm localization of mutant FOXP3, whereas C394Y led to the diffusion of FOXP3 proteins in the whole cell (Fig. 3). FKH mutations changed FOXP3 functions

Hep3BFOXP3 stable cells were generated by picking single clones. In the culture, large cell bodies and multiple nuclei were always observed in the Hep3BFOXP3 clones but not in the control stable clones (Fig. 4A). To rule out the effects caused by clone to clone variations, MCF7 cells, which were previously used in the FOXP3 functional study (9, 14), were transfected with plasmids encoding EGFP or FOXP3EGFP fusion proteins of either WT or mutant FOXP3 (pEgfp, pFOXP3Egfp of pMutFOXP3EGFP-L389P) (Fig. 3). The cells were then selected with G418 and the drug-resistant cells were combined and cultured. In the culture, large cell bodies and multiple nuclei were frequently observed in stable cells that expressed WT FOXP3 fusion protein (MCF7foxE) but not in control stable cells (MCF7EGFP) and mutant FOXP3-expressing cells (MCF7foxEmut). When the cells were grown in 6-well plates and reached 100% confluence, MCF7foxE showed significantly lower cell number (120 ± 8 × 10³) compared with that of MCF7EGFP (333 ± 32 × 10³) and MCF7foxEmut (297 ± 21 × 10³) (Fig. 4B, upper panel). In the culture of MCF7foxE, the medium became acidic much faster than that in the cultures of MCF7EGFP and MCF7foxEmut, indicating FOXP3 overexpression led to a higher metabolic rate in MCF7 cells (Fig. 4C). However these three stable cells demonstrated similar doubling time in culture at subconfluent stage if the medium was changed regularly to guarantee nutrient sufficiency.

To examine whether the tumor-inhibitory function of FOXP3 was influenced by the FKH mutations identified, the EGFP-or FOXP3EGFP-expressing MCF7 stable cells were established (Fig. 5A). The purified cells were harvested, cultured, and injected into SKID mice. The xenograft tumors of MCF7EGFP, MCF7fox3E, and MCF7fox3Emut were monitored for 30 days (Fig. 5B). The volumes of the tumors formed by these types of MCF7 cells were not different until Day 22 and by the end of the experiment (Day 30), tumors formed by MCF7EGFP and MCF7fox3Emut were obviously larger than that by MCF7fox3E, which was barely detectable, suggesting that the inhibitory function of FOXP3 was lost when it was mutated. Similarly, stable cells were also established with HCC Hep3B cells and purified for xenograft tumor growth assay in SKID mice. The xenograft tumors formed by Hep3B cells with FKH mutations (L389P and K393M/E400G) were much larger than that formed by Hep3B cells with wildtype FOXP3, which further supported that mutant FOXP3 lost the tumor-inhibitory ability (Fig. 5C).

FKH mutations influenced the transcriptional activities of FOXP3

RGS1 and ELF1 are two FOXP3 target genes (15–17). The expression of these two molecules was repressed in FOXP3-overexpressing cells (17) and chip-on-chip analysis demonstrated direct binding of FOXP3 on the promoters of these two genes (15). Therefore we cloned their promoters to examine the effect of FKH mutations on the transcriptional capability of FOXP3. It was found that FOXP3 could significantly suppress the activities of RGS1 and ELF1 gene promoters, whereas when FKH mutation L389P was introduced into the FOXP3 ORF, the transcriptional regulatory activity of FOXP3 was greatly attenuated (Fig. 6A, left panel). FOXP3EGFP fusion also exerted a suppressive effect on the target promoters, but the effect was not as robust as that of FOXP3 alone (Fig. 6A, right panel).
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A

Hep3Bct-1
Hep3Bfoxp3-1

Hep3Bct-2
Hep3Bfoxp3-2

75kD
50kD
37kD
25kD
42kD
FOXP3
β-actin

B

MCF7EGFP
(333±32×10^4 /per well)

MCF7foxE
(120±8×10^4 /per well)

MCF7foxEmut
(297±21×10^4 /per well)

Cell number in confluent culture (×10^4 cells/well)

P<0.05

GFP IgG

FOXP3 IgG

C

MCF7EGFP

MCF7foxE

MCF7foxEmut

lower confluency

higher confluency
Similarly, the presence of L389P mutation in the FKH domain resulted in loss of the transcriptional regulatory activity (Fig. 6A, right panel).

**Figure 4. Effects of FOXP3 on cellular features were reversed by FKH mutations.** A, FOXP3 overexpression caused large cell bodies and multiple nuclei in Hep3B stable cells. Hep3B stable cells were generated by picking single clones and two clones of each of control and FOXP3 expressing clones were observed. Arrows, multinucleated cells. *Left panel*, Western blotting showing FOXP3 overexpression in Hep3BFOXP3 clones. *Scale bar* = 50 μm. *B*, FKH mutation reversed effects of FOXP3 in inducing morphological changes of MCF7 stable cells. G418-selected MCF7 cells were combined and subjected to observation. *Upper panel*, representative images showing confluent cultures in 6-well plate. Each cell number represents the mean ± S.D. of counting results of three wells. *Scale bar* = 50 μm. *Lower left panel*, statistical analysis of the number of cells in 3 subtypes of MCF7. *Lower right panel*, Western blotting showing expression of FOXP3EGFP fusion proteins. All test results were generated from three independent experiments. C, FOXP3-overexpressing MCF7 cells have higher metabolic rates than MCF7EGFP and MCF7foxEmut cells. Cells were seeded at lower or higher densities and grown in phenol-red supplemented medium for 48 h before images were taken.

**Figure 5. FKH mutation reversed tumor-inhibitory functions of FOXP3.** Cells were transfected and G418-selected. The resulted G418-resistant cells were then combined and subjected to two rounds of cell sorting to isolate EGFP or FOXP3EGFP expressing stable cells. 10^7 cells were injected into female SKID mice subcutaneously in xenograft tumor growth assay (n = 4). A, representative MCF7 cell images after G418 selection. *Scale bar* = 50 μm. B, the growth of MCF7 xenograft tumors. Two independent experiments were conducted in this work. Data were expressed as mean ± SD.

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**Figure 6A.** FOXP3 was also able to significantly suppress the promoter activity of RGS1 gene in HCC Hep3B cells. However the regulatory power was obviously lower compared with that in 293T
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Cells. It was noted that the expression profile of “classical” HDACs in Hep3B was different from that in 293T and that HDAC inhibitors showed much stronger effects on the promoter activity in Hep3B than that in 293T (18). Thus, our data suggested that HDACs may play roles in attenuating the effect of FOXP3 in Hep3B. This suggestion was supported by the HDAC inhibition experiment on FOXP3-overexpressing cells. Hep3B cells transfected with mutant FOXP3 or WT-FOXP3 were treated with HDAC inhibitor SAHA before the cells were harvested for luciferase assay. SAHA tremendously induced the RGS1 promoter activity in the control vector and the mutant FOXP3-transfected Hep3B, whereas the induction was significantly reduced in WT-FOXP3 transfected cells (Fig. 6B, left panel). As a result, FOXP3 demonstrated much more robust suppressive effects in SAHA-treated cells than in cells without SAHA treatment, and the FKH mutation led to the loss of regulatory capability (Fig. 6B, right panel).

The interaction between FOXP3 and HDAC1 or HDAC2 was previously reported in breast cancer (19) and such an interaction was confirmed in our co-immunoprecipitation assay. Interestingly, the interaction was not affected by the FKH mutation although the mutation altered the subcellular localization of FOXP3 (Fig. S1).

The cytotoxic effects of various HDAC inhibitors were also examined in stable Hep3B cells expressing WT or mutant FOXP3. TSA significantly suppressed the growth of both WT and mutant FOXP3 cells whereas SAHA and valproic acid (VPA) showed the cytotoxic influence on WT FOXP3 cells only but not on mutant FOXP3 cells. Some HDAC inhibitors, such as mocetinostat, sodium butyrate (NaB), and VPA, even showed significant promoting effects on mutant FOXP3 cells. However, all the HDAC inhibitors demonstrated significant suppressive effects on both WT and mutant FOXP3 cells when the proteasome inhibitor MG132 was added (Fig. 6C).

Discussion

FKH structure is critical for FOX protein functions (3). The mutations in this domain usually cause dysfunction of FOX proteins that can lead to severe diseases, such as mutant FOXC1 for anterior segment dysgenesis, mutant FOXC2 for lymphedema, and mutant FOXP2 for speech and language disorder (20–22). The severe IPEX syndrome was found to be caused by FOXP3 mutations that also mainly occurred in FKH domain (23). Mutations of FOXP3 have also been reported in cancers (8, 9), indicating mutation-associated FOXP3 may play roles in cancer development/growth.

In this work, we screened the FKH region for mutations because this is the key region to determine the subcellular localization of FOXP3 and mutations in this region may result in the complete loss of FOXP3 functions (3, 4). We started FOXP3 mutation screening by examining the FOXP3 transcripts because mRNA was the direct template for protein synthesis. We demonstrated that the frequency of FKH mutations in HCC was quite high. Various mutations were detected in about 33% of tumorous tissues but in none of the corresponding nontumorous tissues (Fig. 1 and Table S1). The absence of mutations in the nontumorous samples helped to rule out two factors that might affect the sequencing results, PCR-generated errors and FOXP3 mRNA expressed by the infiltrating lymphocytes. It was reported that FOXP3-positive lymphocytes were also detectable in the infiltrating lymphocytes of both tumorous and nontumorous tissues (24), which was confirmed in this study (Fig. 1C). Therefore, if FOXP3 mutations in tumorous tissues were because of the presence of the infiltrating lymphocytes or PCR-generated errors, similar mutations should have also been found in the nontumorous tissues.

When we compared all the mutation-positive patients with the mutation negative patients, it seemed that the FKH mutation in tumors had no significant association with survival time. However, 17 patients with two or more point mutations in FKH demonstrated significantly poorer prognosis than that of the patients without mutations in FKH. This is possibly because more point mutations will increase the proportion of nonsynonymous substitutions, which leads to protein structure changes and thus the severe damage to its functions.

We have found that FOXP3-overexpressing MCF7 cells demonstrated larger cell bodies compared with the control and the mutant FOXP3-expressing MCF7 cells (Fig. 4B). It has been proposed that cell size plays an important role in cell proliferation and apoptosis and smaller cell size is correlated with cancerous features (25–27). Thus the cell size enlargement caused by FOXP3 overexpression, possibly because of cellular senescence induced by FOXP3 (28), may indicate the loss of cancerous features or capabilities, which may explain that FOXP3-overexpressing cells are more sensitive to some drugs than the control cells and the mutant FOXP3-expressing cells (Fig. 6C). Interestingly, these large cells also appear to be highly metabolic (Fig. 4C), suggesting that more nutrients are needed to support their cellular activities and that they may be difficult to maintain their survival in the competitive or adverse microenvironment.

The existence of FKH mutations in HCC is in line with the reported suppressive functions of FOXP3 in cancers (4, 6, 9). Many mutations caused the change of FOXP3 subcellular local-

Figure 6. FKH mutations influenced transcriptional activities of FOXP3. A, FOXP3 expression in hepatocellular carcinoma 293T cells were co-transfected with luciferase reporter plasmids and plasmids encoding WT/mutant FOXP3 (left panel) or FOXP3EGFP fusion protein (right panel). The Luc reading was normalized with protein concentration. The data are expressed as -fold changes of luc reading of WT control–transfected cells over that of vector control–transfected cells. Each column represents the mean ± S.D. of three independent experiments. B, suppressive effects of FOXP3 on RGS1 promoter were influenced by HDAC proteins in liver cancer cells. Hep3B was co-transfected with luciferase reporter plasmids and plasmids encoding WT/mutant FOXP3. The cells were then treated for 24 h with HDAC inhibitor SAHA before being harvested for Luc assays. The Luc reading was normalized with protein concentration. Each column represents the mean ± S.D. of three independent experiments. C, suppressive effects of FOXP3 on RGS1 promoter were influenced by HDAC proteins in liver cancer cells. Hep3B was co-transfected with luciferase reporter plasmids and plasmids encoding WT/mutant FOXP3. The cells were then treated for 24 h with HDAC inhibitor SAHA before being harvested for Luc assays. The Luc reading was normalized with protein concentration. Each column represents the mean ± S.D. of three independent experiments. D, FOXP3 mutation triggers different responses to HDAC inhibitors in Hep3B cells. Cells were seeded at 5 × 10^3 cells per well of 96-well plates and grown for 24 h. Afterward the cells were treated as indicated for 48 h and analyzed for proliferation rate by MTT. The proliferation rate is expressed as the value of treated cells versus that of the corresponding untreated cells (equals 100%). Each column represents mean ± S.D. of four replicates. ctr, control.
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ization which was critical for its transcriptional activity (Fig. 3). Subsequent experiments demonstrated that the mutations diminished FOXP3 functions in regulating the expression of target genes (Fig. 5) and suppressing the growth of xenograft tumors (Fig. 6). However, the FKH mutation did not affect interactions between FOXP3 and its protein partners HDAC1 and HDAC2 (Fig. S1). Because FOXP3 without the FKH domain is still able to assist its partners to regulate gene expression (29), it would be interesting to know whether the FKH mutations identified in HCC may enable some novel functions for FOXP3 in cancer cells. Interestingly, we have found that the mutated FOXP3 significantly changed the response of HCC cells to some, but not all, HDAC inhibitors compared with that of WT FOXP3. In the presence of the mutated FOXP3, some HDAC inhibitors even promoted the proliferation of HCC cells (Fig. 6), suggesting that FKH mutations not only result in the loss of transcriptional regulation functions, but also endow FOXP3 with some novel oncogenic functions. Although each HDAC inhibitor used in this study exhibits different potencies (IC50) against various HDAC members (30), SAHA is a pan HDAC inhibitor; TSA is against Class I, IIa, and IIb members; VPA against Class I and IIa members; moclentinostat and NaB against Class I members only. Our data showed that among various HDAC inhibitors tested, TSA and SAHA had cytotoxic effects on HCC cells whereas HDAC inhibitors limited to Class I and IIa members (VPA, moclentinostat, and NaB) showed no effects on HCC cells with WT FOXP3 or even promoted the growth of HCC cells with the mutated FOXP3, suggesting the importance of Class IIb HDAC members in the regulation of HCC cell growth. Our results further demonstrated a synergistic interaction on HCC cell growth by the combination of HDAC inhibitors and MG132, a proteasome inhibitor. Although the synergistic anti-tumor effect by HDAC inhibitors and proteasome inhibitors has been reported in cancers (31, 32), to our best knowledge, this is the first report to show this type of the synergistic anti-tumor effect in HCC. Importantly, this synergistic anti-tumor effect is toward HCC cells with or without mutant FOXP3.

We have also examined the sequences of the corresponding regions in genomic DNA and found no mutations, indicating the FKH mutagenesis in HCC occurred in the process of transcription. The implication of FOXP3 transcription errors in cancers has been reported before. Merlo et al. (29) found that in most breast tumors FOXP3 was detected predominantly in the cytoplasm and demonstrated the existence of mutant FOXP3 proteins with altered FKH domains (33). However, FKH mutations in genomic DNA were detected in only 5 of 65 tumors (~ 8%) (9). By comparing the mutation rates in FOXP3 protein and genomic DNA, it is certain that most mutations of FOXP3 proteins are caused by the transcriptional mutagenesis but not derived from mutations that already existed in genomic DNA. Therefore, our result has demonstrated that the transcriptional mutagenesis of FOXP3 can commonly occur in HCC, which frequently causes the dysfunction of FOXP3.

All the mutations were identified as point deletion or substitution mutations that were possibly because of transcription errors (Fig. 1). Transition mutations (G/A or C/T) occurred much more frequently than transversion mutations (A/T or C/G), which is similar to the observations in other transcription error studies (34, 35). However, no prevalent point mutations were detected at high frequencies in the samples, indicating the mutagenesis occurred randomly.

The reported suppressive effect of WT FOXP3 on tumor growth has been confirmed in our work because the FKH mutation in HCC led to the loss of FOXP3 functions in mouse xenograft models. Further work is worth doing to elaborate the significance of FKH mutations in HCC development, and better to incorporate other liver-specific factors, such as hepatitis virus infection, and gene expression profiles, e.g. expression of HDAC proteins (Fig. 6). We found that one of identified FKH mutants totally abolished the suppressive effect of FOXP3 on RGS1 promoters whereas it could only partially attenuate the effect of FOXP3 on ELF1 (Fig. 6A). In addition, the mutations did not cause the loss of interaction between FOXP3 and HDAC proteins (Fig. S1). More interestingly, the FKH mutation is significantly correlated with severe vascular invasion and worse AJCC staging (Table S1), and the FKH mutation in tumors is significantly associated with severe liver cirrhosis (grade C), the leading risk factor for HCC (36). Considering these results with previous reports (29), we can presume that the FKH mutation not only diminishes functions owned by WT FOXP3 but also enables some novel functions for FOXP3 in cancer growth and/or development. These results have indicated that mutations caused by the transcription error play important roles in the growth of HCC, in line with early observations (37).

Transcription errors are induced by compromised fidelity of RNA polymerase which may be reflected by dysfunction or defect of its subunits (38). For example, RPB9 functions to guarantee the transcription accuracy, however, it was found that the expression of its encoding gene POLR2I was down-regulated in cancers (11, 35). In addition, transcription-related mutations was found to be promoted by increased gene expression levels which are commonly observed in cancer cells (36, 37). We have also observed the down-regulation of POLR2I expression in most HCC tumors, particularly in all of the 38 tumors in which FKH transcriptional mutations were observed (Fig. 2B). The coincidental occurrence of FOXP3 mRNA mutation and downregulated POLR2I expression in drug-treated cells (Fig. 2) further supported the correlation between the transcription error and the expression of POLR2I. Therefore, mutations on mRNA but not on genomic DNA in HCC can be very well-explained by the down-regulation of POLR2I that damages the fidelity of RNA polymerase (11, 39).

In conclusion, we have found that mutations in the FKH domain of FOXP3 mRNA frequently occurred in HCC. The mutations were most likely caused by transcription errors rather than genomic DNA mutations. Our results have suggested for the first time that the transcriptional mutagenesis of FOXP3 may play remarkable roles in HCC growth and/or development, however, further work is necessary to elaborate the significance of the mutations on FOXP3 functions.
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Experimental procedures

Tissues and cell cultures

The human HCC tissue specimens (Table S1) were collected after obtaining the written informed consent, and the use of human samples in this study was approved by the joint Chinese University of Hong Kong (CUHK)–New Territories East Cluster Clinical Research Ethics Committee and the studies were abided by the Declaration of Helsinki principles. All patients were enrolled in Prince of Wales Hospital and informed consent was obtained from all patients. Fresh HCC tumor tissues and the corresponding nontumor tissues (>5 cm away from the edge of the tumor tissue) were obtained and then subsequently placed in liquid nitrogen before the analysis. The diagnosis of all HCC cases was pathologically confirmed. All relevant experiments were performed in accordance with the relevant guidelines and regulations. Human liver cell lines Hep3B, C3A, Huh7, and PCL/PRF/5 and human breast cell line MCF7 were purchased from American Type Culture Collection (ATCC, Manassas, VA) and authenticated by ATCC DNA profiling.

Mutation screening

Total RNA extracted from HCC tissues was subjected to reverse transcription using oligo(dT)$_{20}$ primer. The cDNA products were then subjected to a nested PCR with Platinum PCR SuperMix (Thermo Fisher) to amplify FOXP3 open reading frame (ORF): In the first PCR, forward primer 5'-TATTA-GAAAGAGAGGTCTG-3' and reverse primer 5'-TAGTT-CCTCGACTGCTCA-3' were used. In the second PCR, forward primer 5'-TGTCAGTCCACTTCACCAAG-3' and reverse primer 5'-CTTTCCCTTGATCTGGTC-3' were used. The 1.2-kb PCR products were purified with DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany) and reverse transcription using oligo(dT)$_{20}$ primer. The cDNA products were then subjected to a nested PCR with Platinum PCR SuperMix (Thermo Fisher) to amplify FOXP3 open reading frame (ORF). The results were compared with the sequence of NCBI Reference Sequence NM_014009.3 to identify mutations.

To examine mutations in genomic DNA, tissues or cells were processed with DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany) and the yielded DNA was used to amplify the target regions for sequencing. The primers were designed according to NCBI Reference Sequence NM_014009.3 to identify mutations.

RT-qPCR

Total RNA extracted from HCC tissues was subjected to RT-qPCR using the ABI7900HT Real Time PCR system (Applied Biosystems). Four replicate wells were read for each sample. The primers are POLR2I forward 5'-CTCTGCAGTCTAAG-3' and reverse 5'-ACTGAGAGAGGTCTG-3'. Four replicate wells were read for each sample. The primers are POLR2I forward 5'-AGGATCGAGAAGGAGGATCACA-3' and reverse 5'-TGTAACCGCAACTAAGTCATAG-3'.

Generation of FOXP3 constructs and stable cell lines

WT FOXP3 ORF was amplified via RT-PCR from total RNA extracted from human liver tissues and cloned into HindIII/NotI site of pcdNA3.1 (+) vector (Thermo Fisher) to construct WT p3.1FOXP3. To generate p3.1FOX3mut plasmid that bears mutant FKH domain, the mutant FKH was amplified from human liver tissue cDNA, digested with restriction enzymes BspEI/NotI, and then used to replace the WT FKH domain in p3.1FOX3. To construct FOXP3EGFP fusion expressing plasmids pfoxE and pfoxEmut, FOXP3-egfp fusion ORF was generated via a two-step PCR as described previously (38) and cloned into the pEGFPN1 vector (Takara, Shiga, Japan). FOXP3 stably expressing Hep3B cell line was established via pcDNA3.1 or p3.1FOX3 transfection and G418 selection. Single G418-resistant colonies were picked and screened for positive clones via Western blotting. FOXP3EGFP-stably expressing MCF7 or Hep3B cell lines were established via pEGFPN1, pfoxE, or pfoxEmut transfection and subsequently G418 selection. The G418-resistant cells were then subjected to two rounds of cell sorting and the EGFP-positive cells were harvested and cultured.

Xenograft tumor model

All animal experiments were approved by the Animal Experimentation Ethics Committee of CUHK and Guangdong Laboratory Animals Monitoring Institute and conducted in accordance with guidelines. 4-week-old female SKID mice were used for generating xenograft tumors of MCF7 or Hep3B and their stable derivatives, MCF7EGFP, MCF7foxE, MCF7foxEmut or Hep3BfoxE, Hep3BfoxEmut. For MCF7 xenograft tumor assay, four mice were randomly included in each group, whereas for Hep3B xenograft tumor assay, two independent experiments were conducted. The number of mice used in the experiment was determined by Rollin Brant’s Sample Size Calculators with alpha level at 0.05 and the desired power at 0.85 according to differential effects (~3 folds) of wildtyped FOXP3 and mutant FOXP3 on cell growth showed in the present study. The mice were maintained on a 12 h/12 h light/dark cycle and provided with standard chow and water access ad libitum. Xenograft tumor models were generated by injecting 1 × 10$^7$ cells subcutaneously in the dorsal sides near right hind limbs of the SKID mice. The growth of the tumors was monitored daily. At the end of experiments, all the mice were euthanized by quick cervical dislocation and the tumors were collected. All the mice were still active before euthanasia.

Promoter activity assay

The 5-kb promoter region was selected at the upstream of the transcription start site of putative FOXP3 target genes (15, 17) and cloned into the pGL3 Luciferase Reporter Vector (Promega, Madison, WI). The construct was verified by sequencing. Promoter activity assay was performed according to the methods described before (18).

Proliferation assay

Cell proliferation was evaluated with MTT method as described previously (18). Briefly, the cells were seeded at 5 × 10$^4$ cells per well of 96-well plates and grown for 24 h. Afterward, the cells were treated as indicated for 48 h and analyzed for proliferation rate by MTT. Each assay was performed in four replicates.

Statistics

Statistical analysis was performed using SPSS. Significant difference was determined by two-tailed Student’s $t$ test, one-way
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analysis of variance followed by the Student’s t test or chi-squared test.

Data availability
The data that support the findings of this study are available from the corresponding author, upon reasonable request. Additional experimental details are provided in the supporting information.

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