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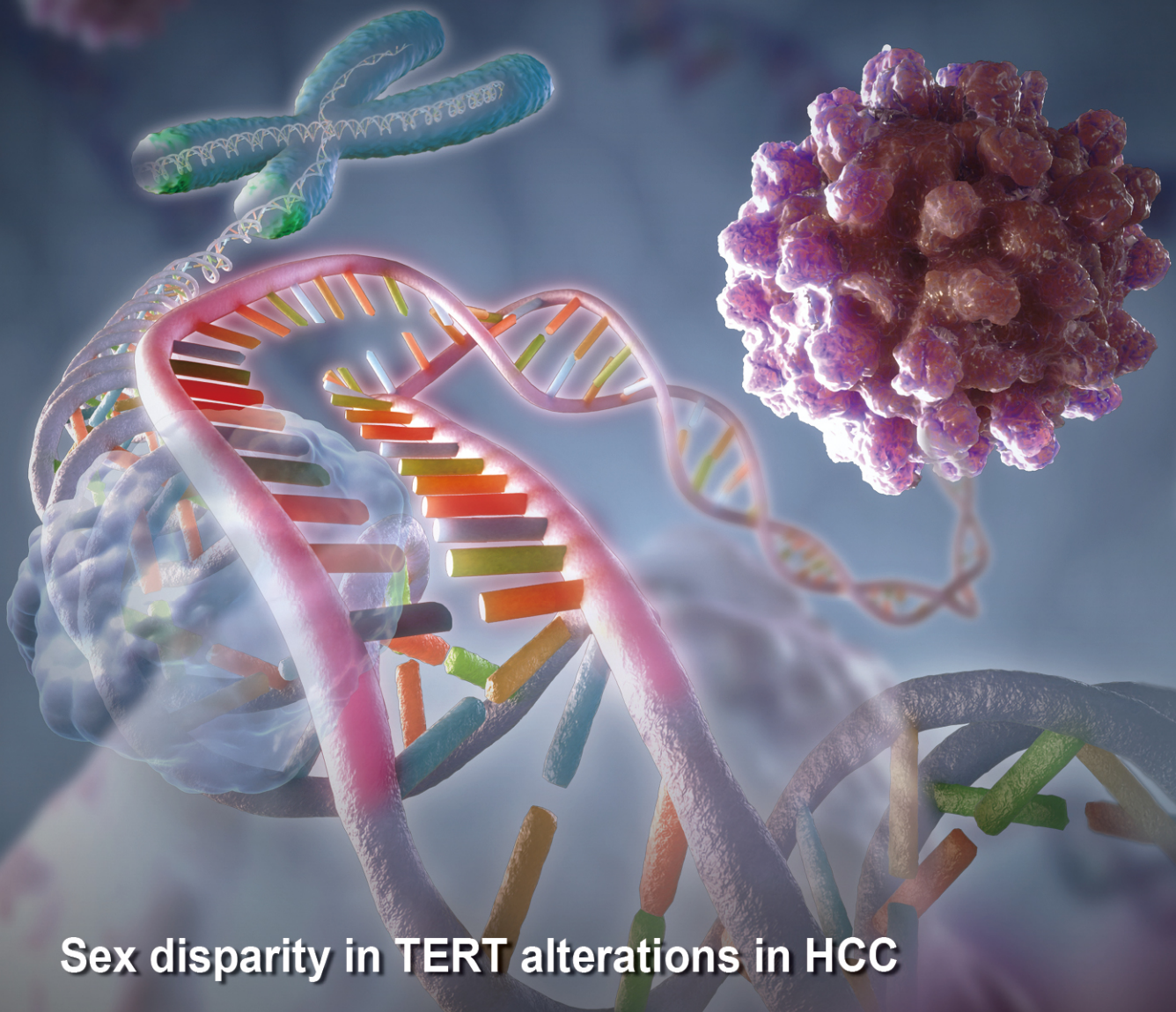

Sex disparity in TERT alterations in HCC

Economic evaluation of hepatitis C elimination
GOLM1 promotes MASH-related gallstone formation
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Original Article

Male preference for *TERT* alterations and HBV integration in young-age HBV-related HCC: implications for sex disparity

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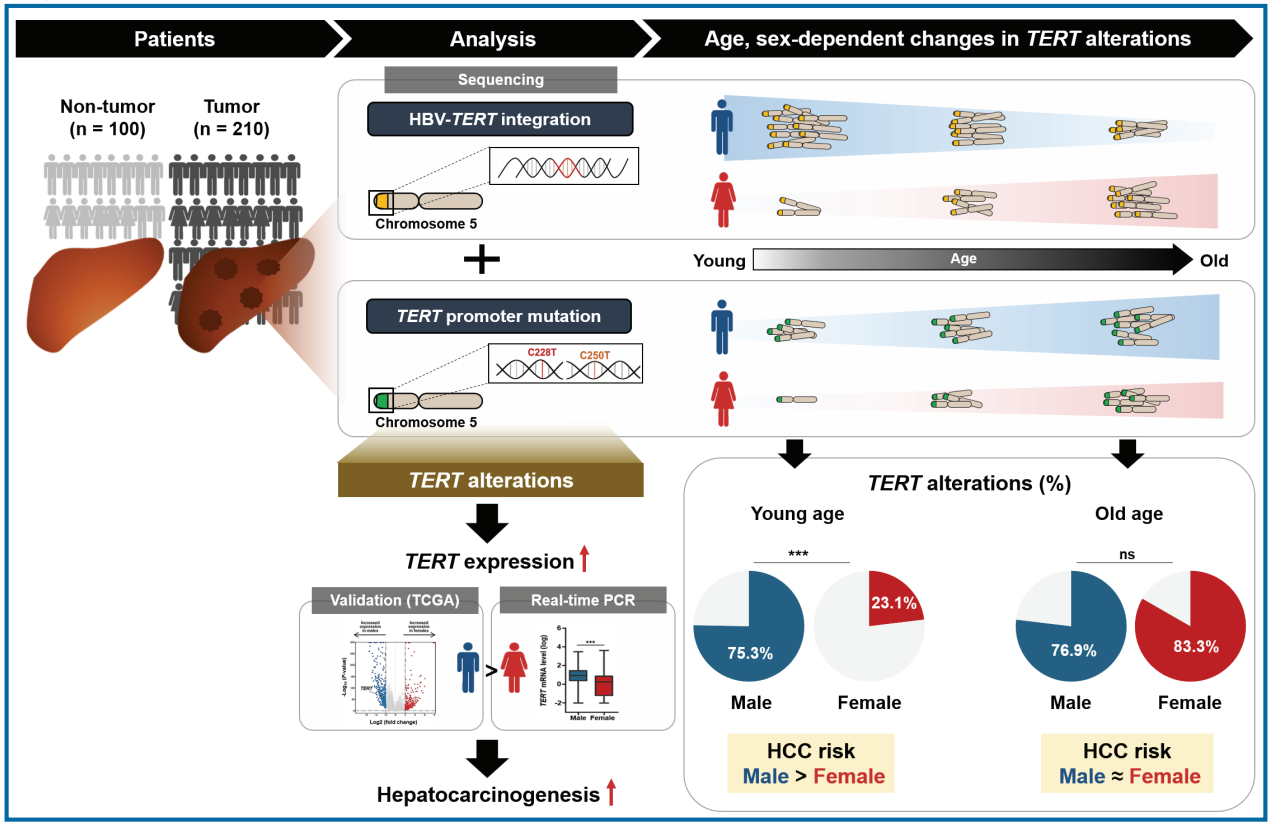
Graphical Abstract



Study Highlights

What is known

- HCC predominantly affects males.
- *TERT* genetic alterations and HBV integration are key contributors to HCC.

What is new

- Younger males have more HBV integrations than younger females.
- Younger males exhibit more *TERT*-promoter mutations and HBV-*TERT* integration compared to younger females.
- This sex disparity in *TERT* abnormalities is observed exclusively in younger, not older, carriers, implicating sex hormones.
- Sex-differential *TERT* alterations are linked to higher *TERT* expression, validated by independent data.

Implications

- Our findings imply molecular characteristics driving higher HCC rates in younger males, offering insights to optimize sex-specific patient care and HCC surveillance strategies.

Background/Aims: Hepatocellular carcinoma (HCC) exhibits significant sex disparities in incidence, yet its molecular mechanisms remain unclear. We explored the role of telomerase reverse transcriptase (*TERT*) genetic alterations and hepatitis B virus (HBV) integration, both known major contributors to HCC, in sex-specific risk for HBV-related HCC.

Methods: We examined 310 HBV-related HCC tissues to investigate sex-specific *TERT* promoter (*TERT*-pro) mutations and HBV integration profiles, stratified by sex and age, and validated with single-cell RNA sequencing (scRNA-seq) data.

Results: Tumors predominantly exhibited *TERT*-pro mutations (26.0% vs. 0%) and HBV-*TERT* integration (37.0% vs. 3.0%) compared to non-tumorous tissues. While *TERT*-pro mutations increased with age in both sexes, younger males (≤60 years) showed marked predominance compared to younger females. Males had significantly more HBV integrations at younger ages, while females initially had fewer integrations that gradually increased with age. Younger males' integrations showed significantly greater enrichment in the *TERT* locus compared to younger females, alongside a preference for promoters, PreS/S regions, and CpG islands. Overall, *TERT* genetic alterations were significantly sex-differential in younger individuals (75.3% in males vs. 23.1% in females) but not in older individuals (76.9% vs. 83.3%, respectively). These alterations were associated with increased *TERT* expression. The skewed *TERT* abnormalities in younger males were further corroborated by independent scRNA-seq data.

Conclusions: Our findings highlight the critical role of *TERT* alterations and HBV integration patterns in the male predominance of HCC incidence among younger HBV carriers, offering insights for future exploration to optimize sex-specific patient care and HCC surveillance strategies. **(Clin Mol Hepatol 2025;31:509-524)**

Keywords: Hepatocellular carcinoma; Telomere; Mutation; Virus integration; Sex

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INTRODUCTION

Hepatocellular carcinoma (HCC) is the most common primary liver cancer and ranks third in cancer-related mortality globally.[1] Major risk factors for HCC include chronic hepatitis B (CHB) or C infection, alcohol, and metabolic factors, with CHB being a prominent cause, particularly in East Asia and Africa. The occurrence of this cancer exhibits a male bias, with a male-to-female ratio of 2–3:1.[2,3] The male-biased HCC occurrence has been even more pronounced in studies of CHB patients, with corresponding ratios ranging from 4 to 6.[3-5]

Hepatitis B virus (HBV) integration into the host genome as a direct oncogenic potential is found in 85–90% of HBV-related HCC tissues. Although this event is not a prerequisite for viral replication, it can promote hepatocarcinogenesis through multiple mechanisms, including cis-mediated insertional mutagenesis, the formation of viral oncoproteins such as PreS2 and HBx, or genomic instability.[6] This integration occurs at an early stage of HBV infection, preceding the emergence of HCC by several decades.[7] However, the precise contribution of viral integration to HCC is yet to be defined.

The genomic landscape of HCC is complex and marked by numerous somatic mutations across a diverse array of genes.[8,9] Among these genetic alterations, the telomerase reverse transcriptase (*TERT*) promoter mutation (*TERT*-pro mutation) is the most frequent alteration, affecting ~60% of HCC patients.[10] *TERT*-pro mutations generate novel binding sites for the E-twenty-six transcription factor family, which contribute to increased telomerase activity and telomere length, ultimately driving hepatocarcinogenesis.[10,11] Recent analyses consistently show that *TERT* is the most common site for HBV integration.[12-15] HBV-*TERT* integration is reported to alter telomerase activity and promotes cellular proliferation, thereby contributing to the oncogenic process.[12,13] These findings suggest an intricate and synergistic interplay between HBV integration and *TERT* dysfunction in HCC development.

It is currently unclear whether the male-dominant nature of HCC risk stems from underlying biologic sex differences or behavioral differences between men and women. While male-predominant smoking and alcohol consumption may be implicated, evidence from HBV-endemic regions indicates a notable difference in HCC incidence between sexes even after adjusting for risk factors.[16] Males typically exhibit an earlier onset and more advanced stage at HCC diagnosis,[17] while a recent analysis of The Cancer Genome Atlas data revealed sex-based differences in mutational profiles, emphasizing the need to consider sex as a biological variable in cancer research.[18] However, there have been limited studies on sex disparity in HCC, and most existing studies have mainly relied on ecologic designs or cancer registry data. Considering the crucial role of telomere-related abnormalities in HCC, unraveling the combined impact of *TERT* mutations and viral insertion-induced carcinogenesis is essential for elucidating the male-biased prevalence in HCC.

To address these gaps, our study explored sex-specific profiles of *TERT*-pro mutations and HBV integration not only in tumors but also in non-tumor tissues by employing an HBV-associated HCC cohort. We conducted age-stratified analyses to better understand the influence of age on sex-specific HCC risk. Additionally, we corroborated our results using single-cell RNA sequencing (scRNA-seq) data obtained from public databases.

MATERIALS AND METHODS

Patients and sample collection

To explore sex differences in HCC risk, the study recruited patients diagnosed with HBV-related HCC at The Catholic University of Korea, Seoul, between February 2017 and December 2021. We analyzed 310 tissue samples, comprising 210 tumors and 100 matched adjacent non-tumor tissues from 210 patients. Of these, 171 HCC tissues were collected from patients aged ≤60, whereas 39 were from those aged >60 years (Supplementary Fig. 1). HCC was diagnosed through histological confirmation, using

Abbreviations:

AFP, alpha-fetoprotein; ALT, alanine aminotransferase; AST, aspartate transaminase; BCLC, Barcelona Clinic for Liver Cancer; BK, breakpoint; EnhI, viral enhancer I; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; HNF-4α, hepatocyte nuclear factor 4 alpha; INR, international normalized ratio; mUICC, modified Union for International Cancer Control; scRNA-seq, single-cell RNA sequencing; *TERT*, telomerase reverse transcriptase; *TERT*-pro mutation, *TERT* promoter mutation; *TERT*-pro, *TERT* promoter

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hepatectomy specimens and liver biopsies.[19] The tissues were immediately frozen in liquid nitrogen and stored at –80°C. This study received approval from the Ethics Committee of The Catholic University of Korea (IRB#No KC-16TISI0436), and all patients provided written informed consent.

NGS technology-based HBV capture assay for HBV integration

Probe-based HBV capture followed by NGS technology was employed to detect HBV integration in tissues, as described previously.[20] Briefly, genomic DNA (gDNA, 1 ug) was fragmented using adaptive focused acoustic technology (Covaris, Woburn, MA, USA), repaired, ligated with an 'A' to the 3' end, and PCR-amplified after ligation of the Agilent (Santa Clara, CA, USA) adaptors. HBV capture utilized 250 ng of the DNA library following the standard Agilent SureSelect Target Enrichment protocol, with hybridization at 65℃ for 24 hours. The purified product was quantified and qualified using quantitative polymerase chain reaction (qPCR) and TapeStation DNA screentape D1000 (Agilent), respectively. Paired-end 100-bp read-length sequencing of the captured DNAs was conducted on the Illumina NovaSeq 6000.

A modified reference combining human (UCSC hg19) and HBV (DQ683578.1) genomes was generated to identify HBV-Human chimeric reads. After mapping paired-end reads to this reference by BWA-MEM, chimeric reads were extracted, and breakpoints (BKs) were predicted from chimeric reads aligned to both human and HBV genomes. HBV BKs with a chimeric read count ≥5 and average mapping quality ≥20 were considered true signal.[20] Approximately 90% of the integration sites identified by our method were previously confirmed by Sanger sequencing.[15]

Sequencing of the *TERT* promoter region

We extracted gDNA from fresh frozen tissue samples using the QIAamp DNA Mini Kit (Qiagen, Hidden, Germany) and conducted direct sequencing. Specific primers targeting –124 bp C>T and –146 bp C>T mutations in the *TERT* promoter were used for PCR amplification: forward 5´-CAGCGCTGCCTGAAACTC-3´ and reverse 5´-GTCCTGCCCCTTCACCTT-3´. PCR was performed on a DNA Engine Tetrad 2 Peltier Thermal Cycler (Bio-Rad, Hercules, CA, USA). DNA sequencing was then conducted on an ABI PRISM 3730XL Analyzer (Applied Biosystems, Foster City, CA, USA).

TERT mRNA expression

RNA was extracted using Qiazol reagent (Qiagen, Hilden, Germany) from frozen tissues following the manufacturer's instructions. cDNA synthesis was conducted using the High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific, Waltham, MA, USA). *TERT* mRNA expression (Applied Biosystems, Hs00972650_m1) was measured by quantitative real-time PCR using Taqman Universal MasterMix (Applied Biosystems, 4324018). Gene expression was calculated using GAPDH (Applied Biosystems, Hs03929097_g1) as an endogenous control.

RNA-seq data collection and processing

For bulk RNA-seq, HCC RNA-seq expression data and matching clinical information were obtained from TCGA repository. For scRNA-seq, the transcriptome data obtained from SRP318499 (https://doi.org/10.1038/s41467-021-24010-1) included female HBV-HCC scRNA-seq and age-matched male data. Gene expression and metadata were reformatted to anndata (10.1101/2021.12.16.473007). Scanpy (v1.9.8) pipeline was used for downstream processing, which involved log-normalization and integration via anndata's concatenate function. Following principal component analysis, Harmony (harmonypy v0.0.5) 57 batch correction was applied based on patient identity. Neighborhood graphs were then derived from the batch-corrected PC axes and projected onto UMAP embeddings.

Marker selection and pathway analysis

Marker gene candidates for each cluster were chosen based on specificity and cluster-wise average expression values, which were maximally normalized to the top-expressing cluster (with its average value set to 1). Genes with a >0.5 difference in expression between the top cluster and the subsequent cluster were chosen as markers. Genes were ranked by the gap value while aiming to avoid lowly expressed genes as markers by applying expression

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criteria (average log-normalized expression value >0.3 and >10% cell expression within the cluster).

For differential expression analysis, *P*-values and fold changes between clusters were computed using the *t*-test on log-normalized gene matrices. Genes that were either expressed in <10% of a cluster or <10 cells, depending on cluster size, were filtered out. Differentially expressed genes were chosen based on log2 fold change >1 and *P*-value <0.05. Pathway analysis utilized EnrichR with GSEApy (v1.0.6).

Table 1. Baseline characteristics of HCC patients

	HCC patients (n=210)	Young (age ≤60 yr) (n=171)	Old (age >60 yr) (n=39)	*P*-value*
Sex				0.335
Male	153 (72.9)	127 (74.3)	26 (66.7)	
Female	57 (27.1)	44 (25.7)	13 (33.3)	
Age (yr)	54.1±8.4	51.5±6.8	65.3±4.6	<0.001
HBeAg seropositivity	51 (24.3)	43 (25.1)	8 (20.5)	0.543
HBV DNA (log IU/mL)	2.5±1.7	2.6±1.8	2.0±1.3	0.083
HBV genotype	C (n=62/62; 100)	C (n=47/47; 100)	C (n=15/15; 100)	>0.999
AST (U/L)	48.5 (3.9–9,000)	57.5 (3.9–9,000)	32.5 (14–177)	0.001
ALT (U/L)	35 (6–2,613)	41 (6–2,613)	26.5 (10–120)	<0.001
Total bilirubin (mg/dL)	0.8 (0.1–29.4)	0.8 (0.1–14.4)	0.7 (0.2–29.4)	0.353
Albumin (g/dL)	3.9±0.7	3.8±0.7	4.0±0.8	0.268
Prothrombin time (INR)	1.2±0.3	1.2±0.3	1.1±0.3	0.469
Liver cirrhosis	98 (46.7)	80 (46.8)	18 (46.2)	0.943
Antiviral therapy	116 (55.2)	93 (54.4)	23 (59.0)	0.603
Child–Pugh class				0.216
A	178 (84.8)	142 (83.0)	36 (92.3)	
B/C	32 (15.2)	29 (17.0)	3 (7.7)	
AFP (ng/mL)	57.8 (0.9–200,000)	59.5 (1.3–200,000)	57.2 (0.9–35,997.1)	0.669
Tumor size (cm)	5.5±4.3	5.8±4.6	3.9±2.3	0.001
Tumor number				0.003
Single	135 (64.3)	102 (59.6)	33 (84.6)	
Multiple	75 (35.7)	69 (40.4)	6 (15.4)	
mUICC stage				<0.001
I	29 (13.8)	23 (13.4)	6 (15.4)	
II	87 (41.4)	60 (35.1)	27 (69.2)	
III	43 (20.5)	40 (23.4)	3 (7.7)	
IV	51 (24.3)	48 (28.1)	3 (7.7)	
BCLC stage				0.010
0	25 (11.9)	19 (11.1)	6 (15.4)	
A	78 (37.1)	57 (33.3)	21 (53.9)	
B	56 (26.7)	46 (26.9)	10 (25.6)	
C	51 (24.3)	49 (28.7)	2 (5.1)	

Values are presented as number (%), mean±standard deviation, or median (range).
AFP, alpha-fetoprotein; ALT, alanine aminotransferase; AST, aspartate transaminase; BCLC, Barcelona Clinic for Liver Cancer; HBeAg, hepatitis B e antigen; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; INR, international normalized ratio; mUICC, modified Union for International Cancer Control; yr, years.
*Comparison between young and old HCC patients.

Definitions

Regarding the human genome, genic region was defined as the combination of promoters (5 kb upstream of the transcription start site), exons (including the 3'-untranslated region), and introns, while the remaining portion was classified as intergenic. HBV integration BKs in the HBV genome were counted while allowing for overlaps within the four open reading frames. Within the *TERT* promoter, hotspot mutations considered were –124 bp C>T and/or –146 bp C>T mutations from the ATG start site. *TERT* genetic alterations encompassed *TERT*-pro hotspot mutations or HBV integration into the *TERT*. To explore age effects, patients were categorized as either younger (≤60 years) or older (>60 years).

Statistical analysis

Data were expressed as mean±standard deviation or median (range). Analyses were carried out using the chi-square test or Fisher exact test for categorical variables, and Student's *t*-test or Mann–Whitney U-test for comparing continuous variables between groups. Correlation coefficient analysis was performed with Spearman methods. A 2-sided *P*-value <0.05 was considered statistically significant. All statistical analyses were performed using SPSS version 20 software (IBM Corp., Armonk, NY, USA) or R software packages.

RESULTS

Patient characteristics

The study included 310 tissue samples (210 HCC and 100 paired non-HCC). Baseline characteristics of the subjects are shown in Table 1. Among the HCC patients, 153 were males and 57 were females, with an average age of 54.1±8.4 years. Of these, 171 were younger patients (≤60 years)–127 males and 44 females, while 39 were older patients (>60 years)–26 males and 13 females. ALT and bilirubin levels were higher in males than in females, while other parameters showed no significant differences. Tumor size, stages, and AFP levels also did not differ between male and female patients (Supplementary Table 1).

Detection of HBV integration BKs in tumors and non-tumors

We identified 4,430 HBV integration sites: 2,318 in tumors and 2,112 in non-tumors. Overall, HBV integration was detected in 85.8% (139/162) of tumors and 94.0% (94/100) of paired non-tumor tissues. Tumors had fewer integration BKs (5 [0–259]) than non-tumors (9.5 [0–274]). However, tumor integrations exhibited higher chimeric read counts than non-tumor integrations (Fig. 1A), suggesting pronounced clonal expansion within HCC. HBV preferentially integrated into promoter regions in tumors, whereas intergenic integration was more common in non-tumor tissues (Fig. 1B). Exclusive genic integration was more prevalent in tumors (33/162, 20.4%) than in non-tumors (9/100, 9.0%) (Fig. 1C). Examination of CpG islands, which are closely related to genome stability, revealed that HBV integrations in tumors were significantly enriched in CpG islands compared to random distribution and non-tumors (Fig. 1D). These findings indicate the oncogenic potential of HBV integration and its preference for specific genomic regions affecting gene expression and stability.

TERT abnormalities in tumors and non-tumors

Additionally, we examined HBV-*TERT* integration and *TERT*-pro mutations in all subjects. HBV-*TERT* integration was significantly more frequent in tumors compared to non-tumor samples (37.0% [60/162] vs. 3.0% [3/100]; *P*<0.001) (Fig. 1E). Notably, *TERT*-pro mutations were exclusively identified in tumors, with no instances observed in non-tumors (26.0% [46/177] vs. 0% [0/51]; *P*<0.001) (Fig. 1F). This distinctive prevalence of *TERT* abnormalities underscores their pivotal role in HCC.

Age-specific pattern of HBV integration in tumors

When examined across age groups, tumor HBV integrations were more prevalent among younger males. Males exhibited a significantly higher overall HBV integration frequency than females until age 60, after which it decreased. Conversely, females initially had fewer integrations, but their integration frequency gradually increased with age until 70 years (Fig. 2A). When analyzed separately by sex, a negative correlation was observed between HBV integra-

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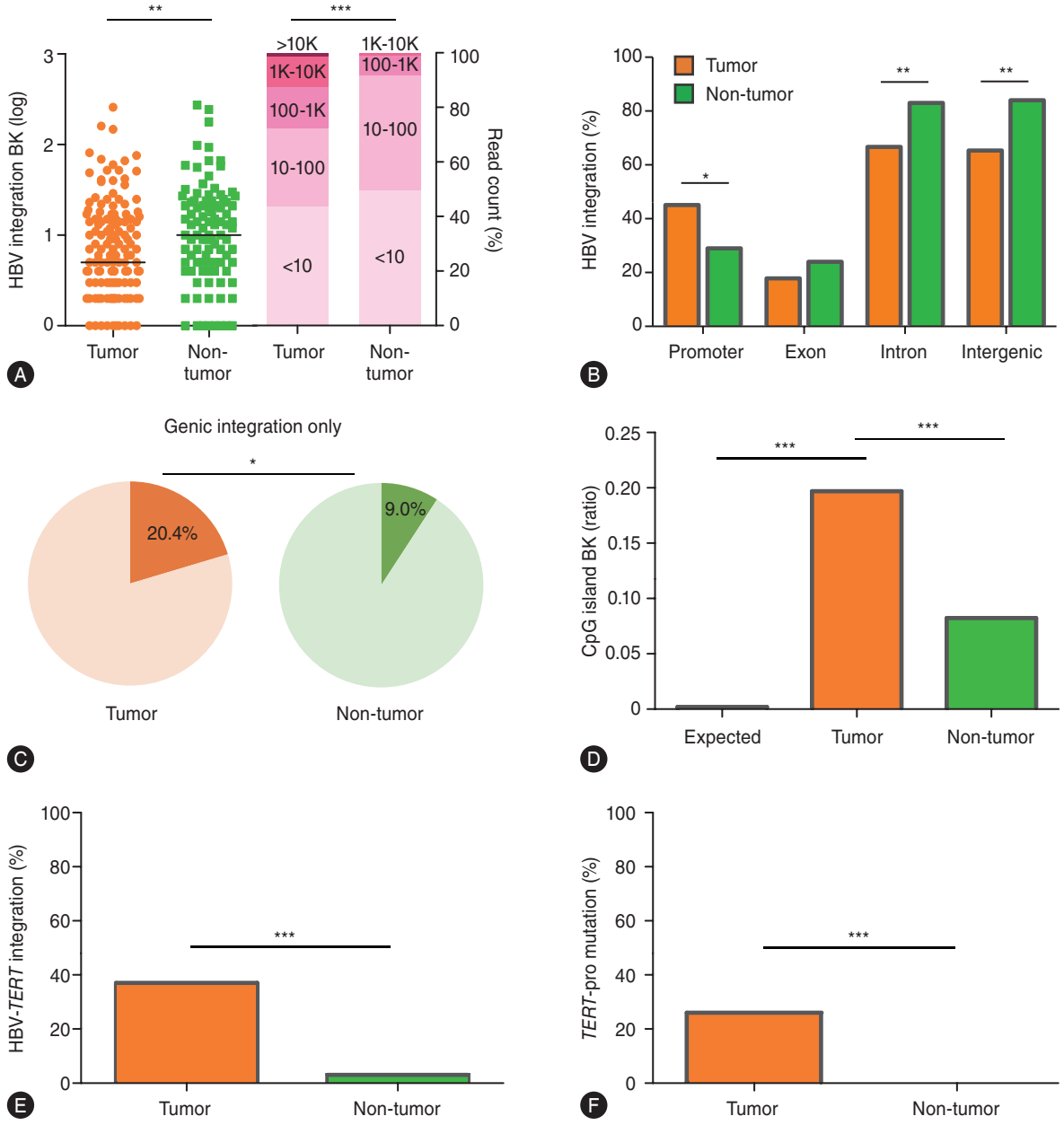

Figure 1. Comparative analysis of HBV integrations and *TERT* genetic alterations in 310 tissue samples, including both tumors and non-tumors. (A) HBV integration BKs and read counts, (B) HBV integration sites in genic and intergenic areas, (C) exclusive genic HBV integration, (D) HBV integration BKs in the CpG island region, percentage of (E) HBV-*TERT* integration and (F) *TERT*-pro mutations within tumor and non-tumor tissues. BK, breakpoint; HBV, hepatitis B virus; *TERT*, telomerase reverse transcriptase. **P*<0.05, ***P*<0.01, ****P*<0.001.

tion BKs and age in males (r=−0.337; *P*<0.001) (Fig. 2B). However, females showed a positive correlation between integration BKs and age, though not significant (r=0.274; *P*=0.079) (Fig. 2C).

Age- and sex-dependent *TERT* alterations in male and female HCCs

We then examined HBV integration and *TERT* abnormalities stratified by sex and age in HCCs. Overall, there was a significant male predominance in HBV-*TERT* integration

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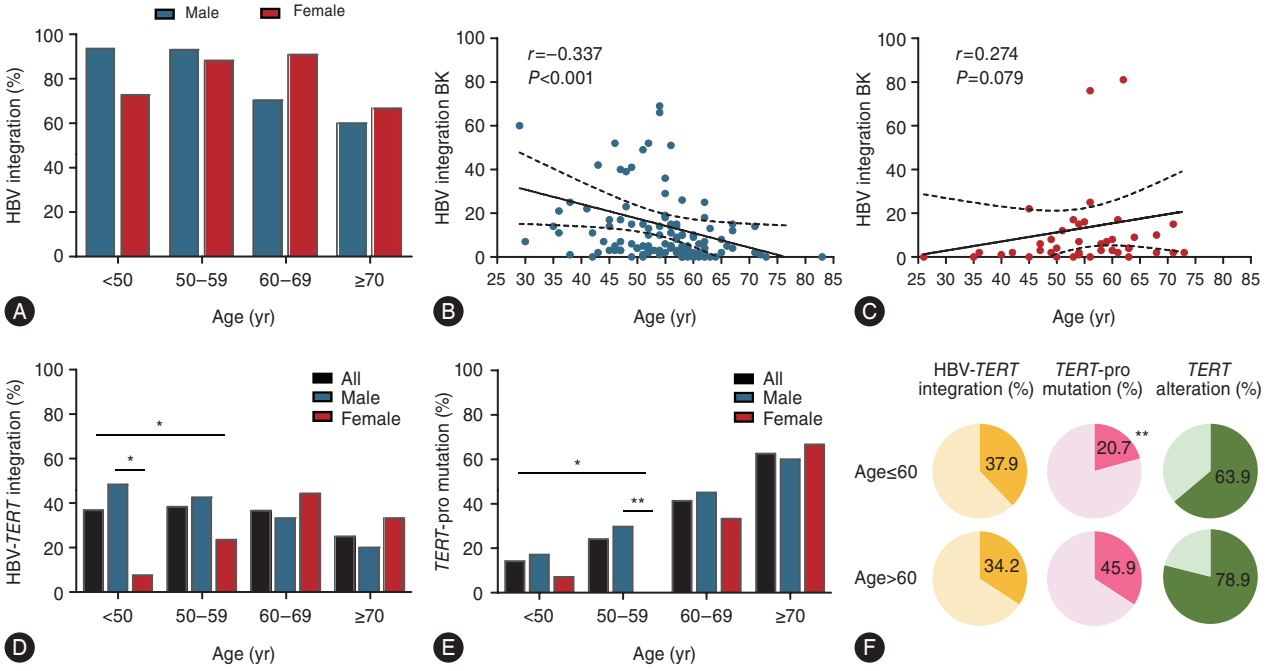

Figure 2. HBV integration in HCC stratified by age and sex. (A) HBV integration rate across age strata. Correlation between age and HBV integration BK in (B) male and (C) female HCCs. Rate of (D) HBV-*TERT* integration and (E) *TERT*-pro mutation by age strata. (F) Comparison of overall rates of HBV-*TERT* integration and *TERT*-pro mutation in individuals under and over 60 years of age. BK, breakpoint; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; *TERT*, telomerase reverse transcriptase; *TERT*-pro mutation, *TERT* promoter mutation. **P*<0.05, ***P*<0.01.

compared to females (41.7% [50/120] vs. 23.8% [10/42]; *P*=0.039). Younger males (<60 years) showed a significantly higher frequency of HBV-*TERT* integrations than females (44.7% vs. 16.7%; *P*=0.006), with males under 50 years exhibiting a notably greater prevalence compared to their female counterparts (*P*=0.016; Fig. 2D). Overall, *TERT*-pro mutations were also significantly more prevalent in males versus females (29.9% [40/134] vs. 14.0% [6/43]) (*P*=0.039), particularly in the 50–59 age group (*P*=0.009; Fig. 2E). Despite similar combined rates of HBV-*TERT* integration across age strata, the frequency of HBV-*TERT* integration was higher in males aged ≤60 years compared to those aged >60 years (Fig. 2D–2F). In contrast, the prevalence of *TERT*-pro mutations increased with age in both sexes (*P*=0.002; Fig. 2E, 2F).

These findings highlight age- and sex-dependent variations in *TERT* abnormalities, particularly notable in younger individuals. Consequently, the younger age group (≤60 years) was chosen for further analyses regarding sex disparities in HCC.

Sex-differential profiles of tumor HBV integration in younger patients

Among younger individuals, HBV integrations were identified in 90.4% (85/94) of male HCCs and 80.0% (24/30) of female HCCs (*P*=0.127; Fig. 3A). Males harbored a higher BK number (median 7 [1,615/94] vs. 3.5 [408/30]; *P*=0.091) and read count per tumor sample than females (Fig. 3B). HBV integration across human chromosomes (Fig. 3C) revealed a male preference for chromosomes 2, 4, and 5, while females favored chromosomes 13, 16, 19, and X (all *P*<0.05). Compared to random events, tumor HBV integration was significantly enriched within CpG islands, with a trend towards greater enrichment in males compared to females (Fig. 3D). Genic regions were preferred for HBV integrations over intergenic regions, with higher frequencies in exons and promoters than expected by random integration. Among genic integrations, there was a preference for promoter integration in males and exon integration in females (Fig. 3E). For the HBV genome, HBV integration into the S and X genes was significantly higher than expected, with a

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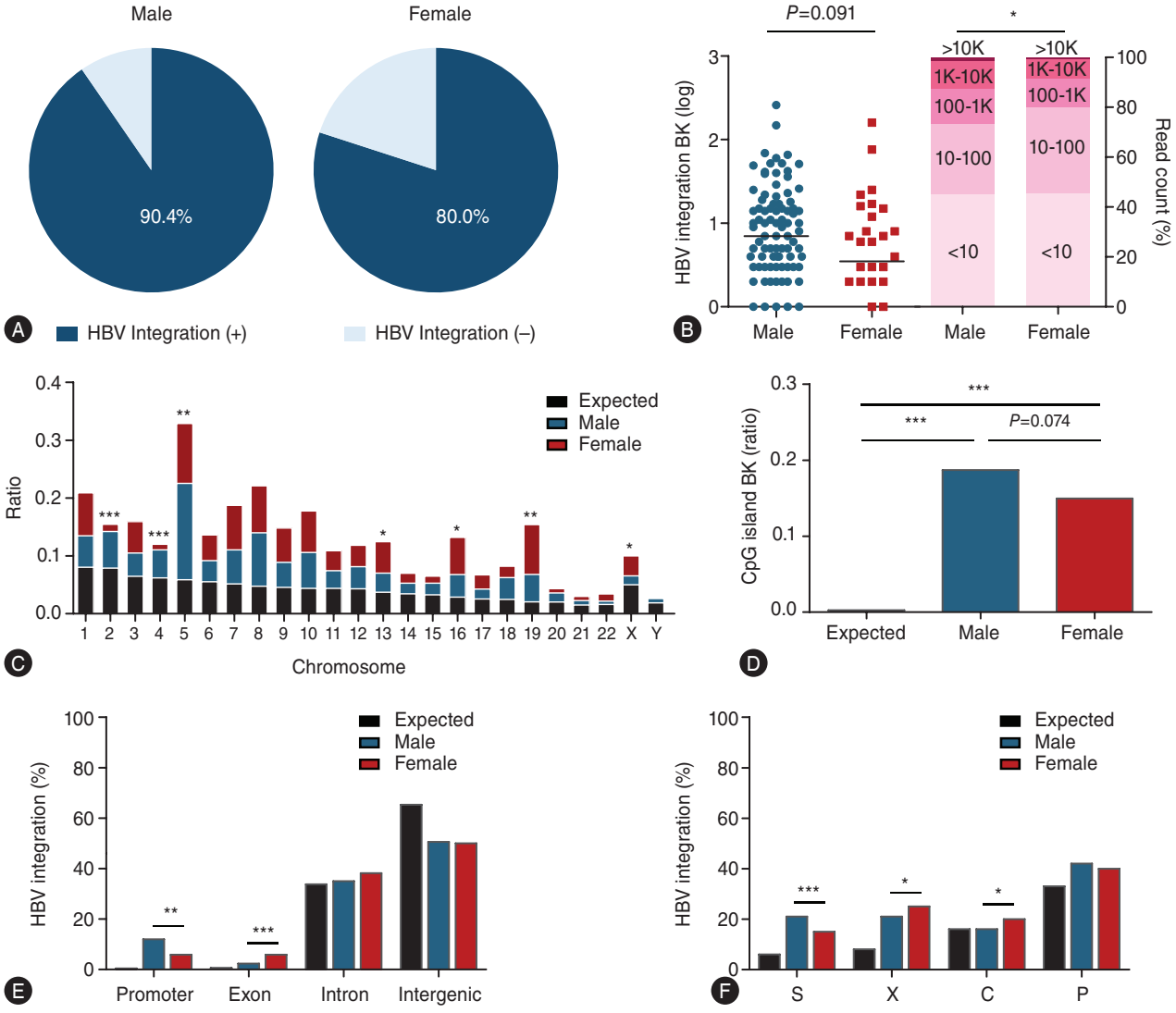

Figure 3. Differential HBV integration patterns in HCC by sex among individuals aged <60 years old. (A) HBV integration rate, (B) HBV integration BK and integration read counts in male and female HCCs. (C) Distribution of HBV integration BK across human chromosomes. HBV integration BK in the (D) CpG island, (E) human genome, and (F) HBV genome. Counts of HBV integration BKs were determined, allowing for overlaps between the four open reading frames. BK, breakpoint; HBV, hepatitis B virus; HCC, hepatocellular carcinoma. *P<0.05, **P<0.01, ***P<0.001.

male preference for the S gene and a female preference for the X and C genes (Fig. 3F).

Location of HBV integration and HBV-*TERT* integration in younger HCC patients

The majority of integration sites clustered within the 1,700–1,900 region of the HBV genome, encompassing diverse functional sequences (Fig. 4A). When surveyed on the human genome, several genes were recurrently integrated by HBV (≥2 samples) (Supplementary Table 2). Among these, the *TERT* promoter was the most frequent integration site, accounting for 37.9% (47/124) of the tumor samples. HBV-*TERT* integrations were predominantly located within ~1 kb upstream of the transcription start site (Fig. 4B). Figure 4C shows the profiles of intragenic locations for recurrent HBV-integrated genes in tumor samples. Integration into the *TERT* locus predominantly occurred within promoters, while integration sites for other genes were largely within introns.

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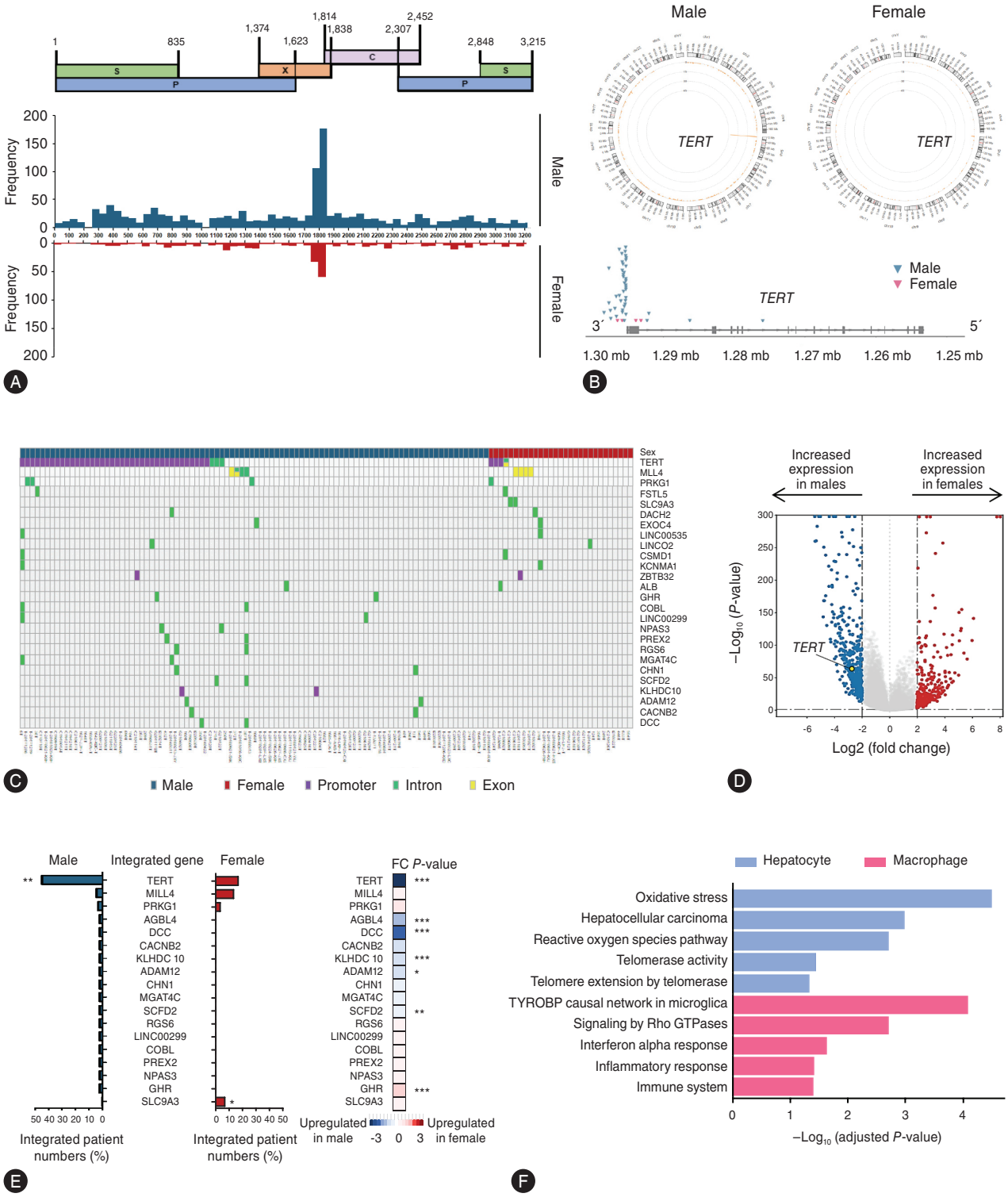

Figure 4. (A) Location, frequency, and read counts of HBV BKs in male and female HCCs. (B) Distribution of integration sites in the human genome and genomic locations of HBV-*TERT* integration BKs for male and female HCCs. (C) Intragenic locations of all recurrent HBV-integrated genes in HCCs. (D) Volcano plot depicting differentially expressed genes in the single-cell transcriptomic analysis of male (blue dots) and female (red dots) HCCs. The *TERT* gene is highlighted. (E) Sex-specific recurrent HBV-integrated genes with their respective differential expression between sexes. (F) Functional enrichment analysis of male-dominant genes. BK, breakpoint; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; *TERT*, telomerase reverse transcriptase. *P<0.05, **P<0.01, ***P<0.001.

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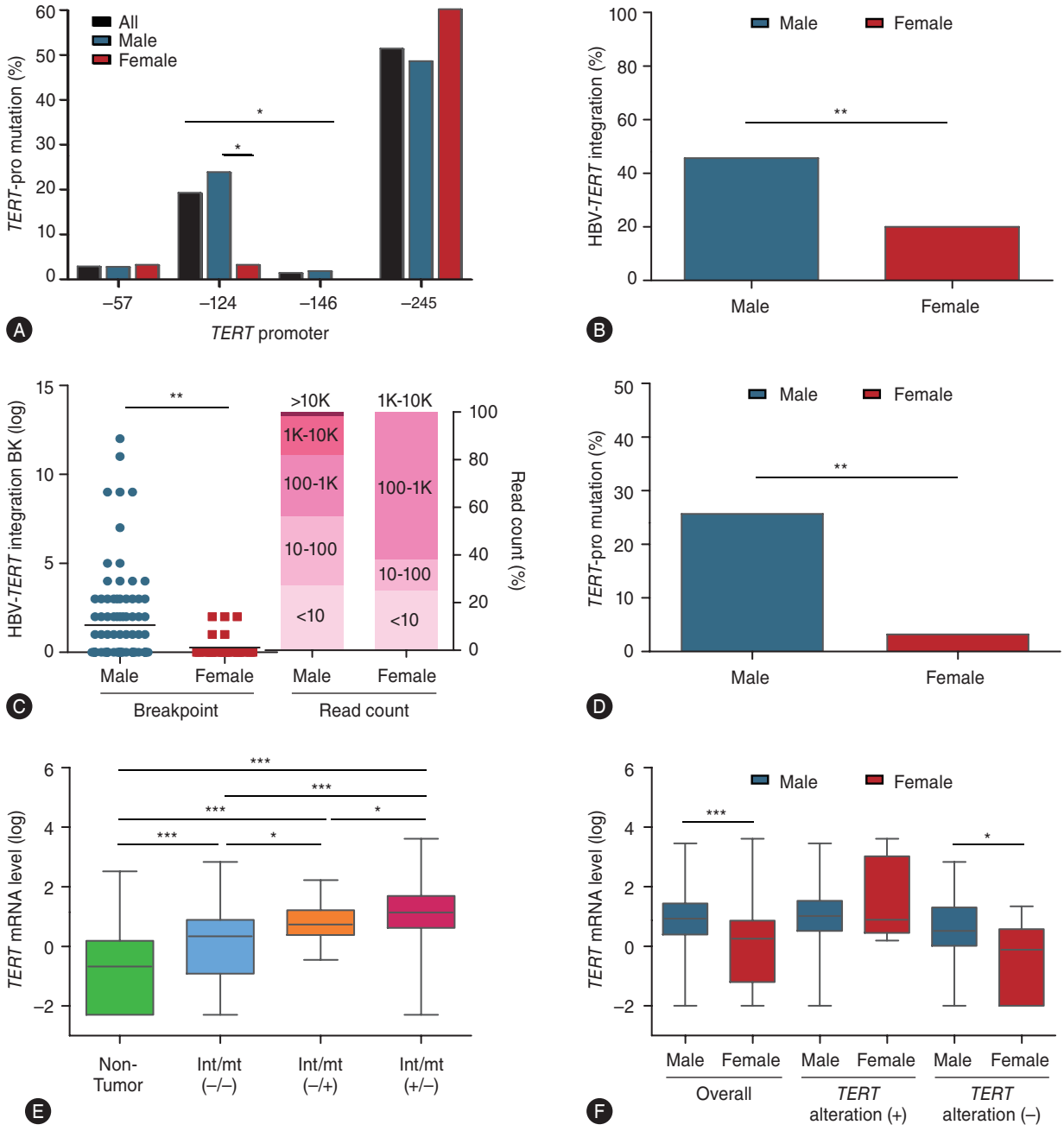

Figure 5. Comparative analysis of (A) *TERT*-pro mutations at different promoter loci, (B) HBV-*TERT* integration rate, (C) HBV-*TERT* integration BK and read count, (D) *TERT*-pro mutation rate, (E) *TERT* mRNA expression levels corresponding to each *TERT* status (integration/mutation), and (F) *TERT* levels according to *TERT* alterations in younger male and female HCCs. Int, HBV integration; mt, *TERT*-pro mutation; *TERT* (+)/(–), with/without *TERT* alterations. BK, breakpoint; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; *TERT*, telomerase reverse transcriptase; *TERT*-pro mutation, *TERT* promoter mutation. **P*<0.05, ***P*<0.01, ****P*<0.001.

Functional enrichment analysis of sex-specific and recurrent HBV-integrated genes in younger HCC patients

To validate the findings regarding sex-differential HCC, we performed sex-differentially expressed gene analysis using publicly available scRNA-seq data from SRP318499 with HBV-HCC. In accordance with the aforementioned observations, we confirmed that males exhibited significantly higher expression of *TERT* (Fig. 4D). Among the sex-spe-

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cific genes with recurrent HBV integrations, *TERT* also emerged as the predominant gene showing sex-specific integration, coupled with markedly differential expression between sexes (Fig. 4E). Moreover, gene ontology analysis revealed significantly enriched *TERT*-related pathways in hepatocytes and inflammation pathways in macrophages, particularly among male subjects (Fig. 4F).

Characterization of *TERT*-pro mutations in younger patients

The two *TERT*-pro hotspot mutations, –124C>T (C228T) and –146C>T (C250T), were identified in 27 (19.3%) and 2 (1.4%) of the tested HCC samples (n=140), respectively, but were not detected in non-tumor samples. Notably, 93.1% (27/29) of the hotspot mutations were the –124C>T mutation, which was significantly more prevalent in males than in females. There were no instances of both *TERT*-pro mutations occurring concurrently, indicating their mutually exclusive nature. Other mutations, including the –138/–139CC>TT tandem mutation, –57A>C mutation, and 245T>C polymorphism, which have been sporadically documented as somatic variations in various cancers,[21] exhibited negligible or no discernible differences between males and females in our analysis (Fig. 5A).

Age-dependent sex disparity in *TERT* genetic alterations in HCC

We analyzed the sex disparities in *TERT* alterations separately in the younger (≤60 years) and older (>60 years) groups. Younger males exhibited a significantly higher prevalence of HBV-*TERT* integration compared to females (44.6% [42/94] vs. 16.7% [5/30]; *P*=0.006) (Fig. 5B), along with significantly increased integration BKs and read counts within the *TERT* locus (Fig. 5C). They also had a significantly higher frequency of *TERT*-pro mutations than females (25.7% [28/109] vs. 3.2% [1/31]; *P*=0.006) (Fig. 5D). Notably, HBV-*TERT* integration and *TERT*-pro mutation were mutually exclusive. We then examined *TERT* mRNA expression according to HBV-*TERT* alterations. Both HBV-*TERT* integration and *TERT*-pro mutations led to higher *TERT* expression compared to cases with no alterations or non-tumors, with tumors harboring HBV-*TERT* integration showing stronger *TERT* expression than those with *TERT*-pro mutations (Fig. 5E). Overall, males showed significantly higher *TERT* expression than females, including those without *TERT* alterations. However, females harboring *TERT* alterations reached comparable expression levels to males (Fig. 5F). No such patterns were observed in older patients (Supplementary Fig. 2).

The overall incidence of *TERT* genetic alterations was observed in 76 (63.9%) younger and 30 (78.9%) older individuals. Among younger patients, males exhibited a significantly higher frequency of HBV-*TERT* integration, *TERT*-pro mutations, and overall *TERT* genetic alterations (75.3% [70/93] vs. 23.1% [6/26], *P*<0.001) than females. Notably, this male predominance in *TERT* alterations remained significant even after adjusting for tumor stage (Supplementary Fig. 3). Conversely, no significant differences in *TERT* genetic alterations were observed between older male and female patients (76.9% [20/26] vs. 83.3% [10/12], *P*=0.652) (Fig. 6A). Overall, age and sex were associated with *TERT* alterations in HCC, with males being the key determinant in younger individuals (Supplementary Table 3).

When analyzed in the TCGA database, *TERT* emerged as a sex-specific differentially-expressed gene, showing higher expression in males versus females among younger patients, while no such distinction was observed among older patients (Fig. 6B). Figure 6C shows a comprehensive overview of *TERT* genetic alterations and mRNA expression in tumors, revealing markedly distinct patterns stratified by age and sex. These findings underscore a significant sex-specific bias in *TERT* abnormalities contributing to the disparity in HCC occurrence, particularly favoring younger males.

DISCUSSION

Sex is a key factor in the development of various cancers including HCC.[22,23] In our study, we conducted a sex-specific genetic analysis of HBV-related HCC, focusing on *TERT* abnormalities. Our findings revealed a noteworthy discrepancy in *TERT*-pro mutations between male and female HCC cases, with a significantly higher prevalence in younger males. Moreover, a substantial younger male predominance was observed in HBV-*TERT* integration. These *TERT* genetic alterations were associated with cancer-promoting biological functions, including *TERT* overexpres-

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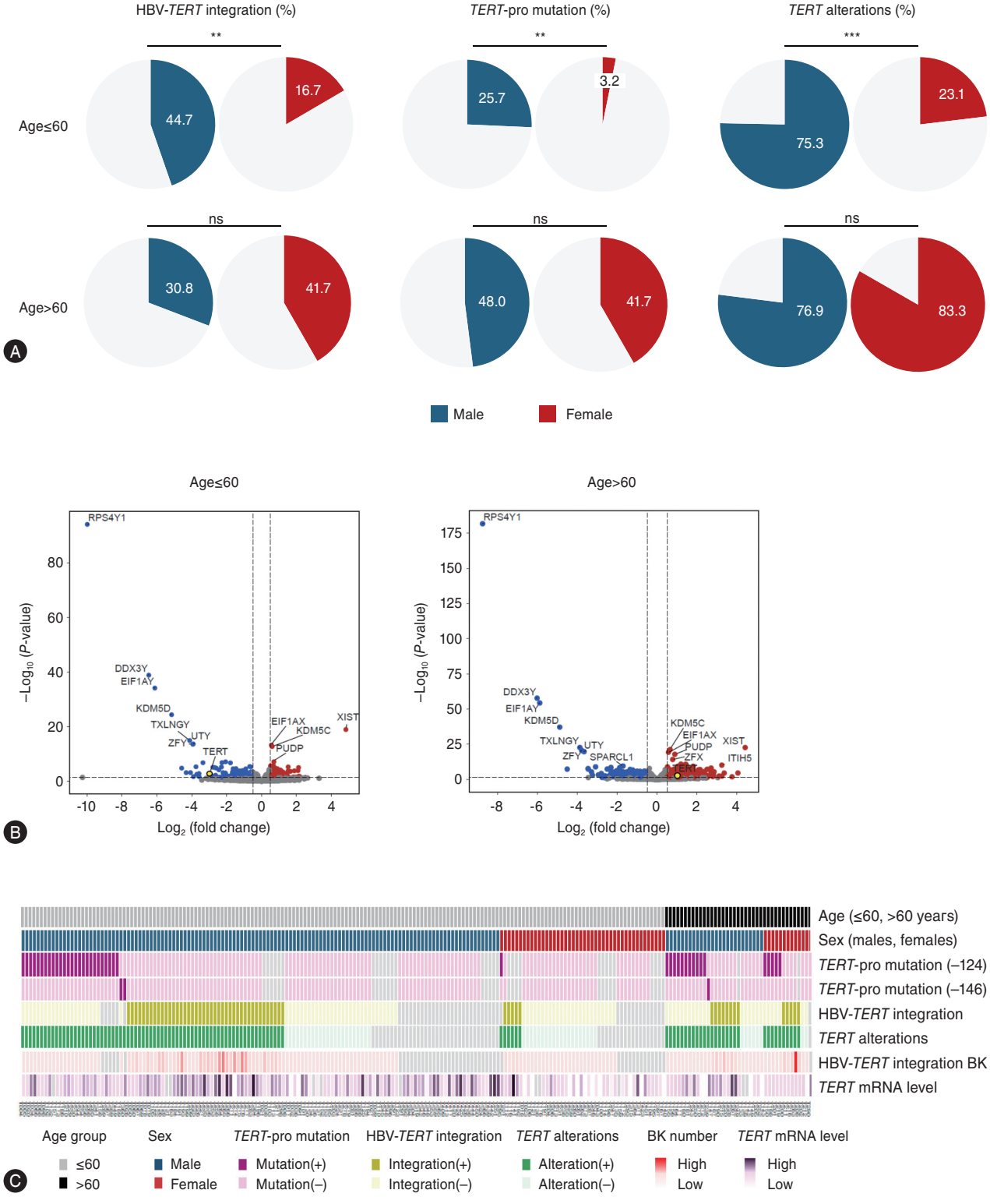

Figure 6. (A) Comparison of HBV-*TERT* integration and *TERT*-pro mutation rates between sexes in individuals under and over 60 years old. (B) Sex-specific differentially-expressed genes in TCGA-HCC database. *TERT* showed differential expression only in younger patients, not in older ones. (C) Schematic view (heatmap) of *TERT* genetic alterations and expression by sex and age in HBV-related HCCs. BK, breakpoint; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; ns, not significant; *TERT*, telomerase reverse transcriptase; *TERT*-pro mutation, *TERT* promoter mutation. ***P*<0.01, ****P*<0.001.

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sion, genome instability, and aberrant oncogenic signaling pathways. Our findings highlight the crucial role of *TERT* genetic alterations, including *TERT* mutations and HBV integration, in contributing to the earlier and more prevalent onset of HCC in males.

Our examination of HBV integration patterns found higher read counts in tumors compared to non-tumors, indicating clonal expansion contributing to HCC tumorigenesis. Tumor integrations were notably enriched in promoter regions and the HBV genomic area for PreS/S proteins, suggesting direct HBV-oncogenic potential. The most frequent integration site was between HBV genome nucleotides 1,700 to 1,900, encompassing HBx (C-terminus). Given the recognized functions of HBx and HBs oncoproteins in HCC, these results further support the carcinogenic potential of HBV integration in HBV-related HCC.

Our findings reveal distinct HBV integration profiles between males and females. Males showed a significant preference for integration into chromosome 5, coinciding with a higher prevalence of HBV-*TERT* integration. Male integrations were also more enriched in CpG islands, which are linked to genome stability, as well as in the PreS/S regions of HBV and promoters, reflecting the higher occurrence of HBV-*TERT* pro integration. Importantly, our results highlight age-dependent, sex-specific patterns of HBV integration. Males exhibited a significantly higher frequency of integration than females until age 60, after which it decreased. Conversely, females had a lower initial frequency that gradually increased with age (Fig. 2). These patterns intriguingly align with the pronounced sex disparity in HCC among younger patients, which diminishes in the elderly,[4] as sex hormone levels decline.

One key finding is the significantly higher prevalence of *TERT*-pro mutation in younger males versus females (25.7% vs. 3.2%). The *TERT* promoter, as a gatekeeper, reportedly undergoes the earliest and most common genetic mutation in hepatocarcinogenesis.[10,24] Interestingly, these mutations were not detected in non-tumor liver in our study. These findings correspond to previous data showing *TERT* mutations in 0% of cirrhotic livers, 6–19% in precancerous nodules, and 61% in early HCCs,[8] again supporting the pivotal role of *TERT*-pro mutation in HCC. Our results were further corroborated by scRNA-seq analysis, which showed elevated *TERT* expression in male HCCs. Thus, these observations unveil a skewed sex-specific pattern of *TERT*-pro mutations as a key driver of HCC, supporting the male predominance in its development.

Additional noteworthy results include the substantial enrichment of HBV integration into *TERT* promoter in younger males versus females (44.7% vs. 16.6%). Our NGS assay revealed several genes with recurrent HBV integrations, with the *TERT* promoter being the most frequent integration site in tumors, in agreement with previous studies.[12-15] These integrations were reportedly associated with increased expression of the proximal gene, which is presumably driven by viral elements.[6,14] Indeed, we found the strongest *TERT* expression in tumors with HBV-*TERT* integration, surpassing the levels observed with *TERT*-pro mutations only or without any *TERT* alteration, consistent with previous reports.[25,26] Given the reported lower frequency (20–30%) of *TERT*-pro mutations in HBV-related HCC compared to that (~60%) in non-HBV HCC,[11] it seems plausible that HBV insertion into the *TERT* promoter emerges as a vital genetic feature, which is strongly implicated in liver carcinogenesis among HBV carriers lacking *TERT*-pro mutations.

Overall, *TERT* genetic alterations demonstrate a striking male predominance in younger individuals (75.3% vs. 23.1%), with higher rates of both *TERT*-pro mutations and *TERT* integrations compared to their female counterparts; however, this difference diminishes in the elderly (76.9% vs. 83.3%) (Fig. 6). These results suggest that *TERT* alterations significantly influence sex-differential HCC risk in younger patients, while carcinogenesis in the elderly may be influenced by additional factors such as behavioral or metabolic causes. Indeed, HCC occurrence reportedly tends to rise among elderly women.[4,27] Emerging evidence indicates that sex hormones contribute to this discrepancy. Androgens activate, while estrogens suppress, *TERT* transcription by targeting integrated HBV within the *TERT* gene through conserved androgen and estrogen responsive elements; both effects depend on hepatocyte nuclear factor 4 alpha (HNF-4α), a key HBV transcription activator.[25] Androgens also enhance *TERT* transcription via the *TERT*-pro –124C>T mutation, facilitating GA-binding protein transcription factor subunit alpha binding to the mutated site.[25] Furthermore, androgens increase HBV activity via the androgen responsive element within viral enhancer I (EnhI),[28] while estrogens repress HBV transcription by upregulating estrogen receptor alpha, which modifies HNF-4α binding

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to EnhI.[29] This heightened inflammation from increased HBV activity in males may lead to greater hepatocyte damage and regeneration, thereby elevating the risk of malignant transformation. Our single-cell transcriptomic analysis of macrophage populations revealed an inflammatory signature in males, underscoring the potential role of inflammation-prone microenvironments in increasing HCC risk under *TERT* dysfunction in young males.

Our study has limitations. It focused solely on sex-differential HCC incidence, without addressing disparities in treatment response or outcomes, and examined only *TERT* genetic alterations, omitting other driver genes. We also did not investigate other genetic or epigenetic factors that may contribute to sex disparities, nor did we conduct functional studies in cell lines or animal models to elucidate the mechanisms by which *TERT* alterations and HBV integration contribute to hepatocarcinogenesis, particularly in a sex-specific manner. These aspects warrant further investigation in future studies. Additionally, our findings require validation across different races and ethnicities, and the potential influence of anti-androgen therapy on HCC in males remains unexplored. Nevertheless, this study provides a comprehensive exploration of *TERT* abnormalities related to sex disparity in HCC by analyzing *TERT*-pro mutations and HBV integration in an etiology-matched, age-stratified cohort, with findings validated through an independent dataset, thereby enhancing the reliability of our results.

In conclusion, young-age HBV-related HCC exhibits a marked predominance of *TERT* genetic alterations in males compared to females, a disparity not observed in the elderly. This study underscores the critical role of *TERT* alterations and HBV integration patterns in driving sex disparities in HCC among younger HBV carriers. Our findings imply molecular characteristics underlying the higher occurrence of HCC in younger males, offering insights for future exploration to optimize sex-specific patient care and HCC surveillance strategies.

Authors' contribution

Jeong Won Jang designed the experiments and analyzed the data; Ji Won Han, Heechul Nam, Pil Soo Sung, Sung Won Lee, Jung Hyun Kwon, Si Hyun Bae, Jong Young Choi, and Seung Kew Yoon performed acquisition of data; Jeong Won Jang, Jin Seoub Kim, Hye Seon Kim, and Kwon Young Tak carried out analysis and interpretation of data; Jin Seoub Kim and Hye Seon Kim performed the experiments; Jin Seoub Kim and Jeong Won Jang wrote the manuscript.

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Conflicts of Interest

The authors have no conflicts to disclose.

SUPPLEMENTARY MATERIAL

Supplementary material is available at Clinical and Molecular Hepatology website (http://www.e-cmh.org).

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