Upregulation of the oestrogen target gene SIX1 is associated with higher growth speed and decreased survival in HCV-positive women with hepatocellular carcinoma

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Abstract. The male/female ratio of patients with hepatocellular carcinoma (HCC) is often unbalanced towards the male sex, indicating a sex predisposition for HCC development. A possible explanation may be attributed to different hormonal statuses, including the pro-inflammatory action of androgens in men and the protective effects of oestrogen against excessive inflammation in women. Although several studies have studied gene expression in patients with HCC, very few have attempted to identify features that could be distinctive between male and female patients. The present study aimed to identify distinctive signalling mechanisms between men and women that may be associated with HCC progression. The present study analysed a detailed microarray database that was obtained from the prospective study of 78 patients with HCC to study gene expression according to sex. In addition, the present study aimed to evaluate whether the differentially expressed genes were known oestrogen targets. Moreover, RNAs from the HCC cohort were evaluated for microRNA (miRNA/miR) expression, and a relationship between miRNA and gene expression according to sex was investigated. One gene, sineoculis homeobox homolog 1 (SIX1), which is known to be an oestrogen target gene, was revealed to be highly upregulated in hepatitis virus C (HCV)-positive female patients with HCC but not in HCV-positive male patients. In addition, SIX1 upregulation had a significant relationship with tumour growth speed (assessed as tumour doubling time in two CTs performed 6 weeks apart) and survival (P=0.009 and P=0.042, respectively) in female patients only. Furthermore, SIX1 upregulation was related with miR-421 and miR-9-5p only in male patients; however, in female patients, SIX1 upregulation had a direct relationship with miR-181b, miR-503-5p and miR-125b (miRNAs with potential oncogenic capacity), and an inverse correlation with miR139-5p, miR-26b, let7c-3p and let7c-5p (putatively oncosuppressive microRNAs). These data suggested a distinctive model for liver carcinogenesis in HCV-positive women, with downregulation of protective mechanisms against tumour progression and the activation of potential oncogenes, in relation to the oestrogen target gene SIX1. (IRB10/08_CE_UniRer; ClinicalTrials ID: NCT01657695).

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Introduction

The most malignant tumour of the liver is Hepatocellular carcinoma (HCC). The male/female rate in HCC is reported to be constantly unbalanced towards the male sex, with more or less marked differences, depending on the aetiology, with Hepatitis B being characterised by more marked imbalance than Hepatitis C (1,2). A consequence of this constant imbalance is that HCC is the third or fourth most frequent solid neoplasm in men for incidence and mortality while in women it is not even among the top 10 (3). Possible explanations for this finding, in addition to those indicated above, have been put forward,

most of them centred on the different hormonal statuses: pro-inflammatory action of androgens in males (4,5), oestrogen protection against excessive inflammation in females offered by the prolonged fertile period (6,7). In addition to the epidemiological and clinical data, the relevance of hormonal status had been demonstrated experimentally as well. In a seminal study, Naugler *et al* (8) showed that the oestrogen-mediated inhibition of Interleukin (IL)-6 production by Kupffer cells reduced liver cancer risk in females. While the suggestion put forward by the authors that these findings might be used to prevent HCC in males cannot be easily applied to the clinical practice since it would imply the feminization of males, the importance of this demonstration remains unambitious. These data clearly indicate that a different gender predisposition to HCC development exists. However, although several studies have investigated gene expression in patients with HCC, only very few, if at all, have evaluated transcriptomic characteristics in relation to sex and the possible relationship between the altered genes with oestrogens in the attempt to identify features that could be distinctive between males and females. In a prospective study of a very well characterised cohort of patients with HCC at first diagnosis, we performed an exhaustive transcriptomic analysis, which allowed us to identify a neoangiogenic transcriptomic signature able to accurately identify rapidly growing and severe HCC cases (9).

In the current study, we have explored the annotated HCC database of the prospective study to understand the differences in gene expression in relation to sex. We found a low number of genes differentially expressed between males and females. An even lower number of genes was differentially expressed in the subgroup of the HCV-positive subjects. Of these genes, sineoculis homeobox homolog 1 (SIX1), ADH1C, and GPR19 were found to be possible oestrogen targets (10). However, only SIX1 was found in HCV-positive women that have a significant correlation with both tumour growth speed and survival. Thus, we have explored the relationship between SIX1 and the clinical, pathologic, and transcriptomic features of HCV-positive patients with HCC in relation to sex as this could yield pertinent indications on various mechanisms of liver carcinogenesis between males and females.

Materials and methods

Patients. We had identified 78 patients at first diagnosis of HCC in a previously reported prospective study on patients with liver cirrhosis on surveillance for HCC (9). After identification of a suspect liver lesion, patients underwent two computed tomography (CT) scans with ad hoc protocol at baseline and after six weeks (no therapy in between) to define the doubling time of tumour size and imaging traits. HCC doubling time (DT) was calculated as t x log2/logV1-logV0. At baseline, after acquiring informed consent, they underwent paired HCC/surrounding-tissue biopsies for microarray analysis (Agilent Whole Human Genome Oligo Microarrays), histochemical (Ki67, CD34, e-cadherin), and histologic evaluation [Edmondson-Steiner (E-S) grading, inflammatory intensity]. Survival, disease-free survival after down-staging and transplant-free survival (Kaplan-Meier) were analysed in relation to imaging and molecular data. A transcriptomic signature capable of separating aggressive HCC from bland HCC was identified. Detailed data are reported in (9).

For the present study, microarray data were re-analysed by bivariate regression analysis according to sex; the genes' differential levels of expression were recorded. The relationship between gender-related expression and transcriptomic signature was also evaluated. We also verified whether the differentially expressed genes between females and males were known oestrogen targets (10). As the females were HCV-positive only, further analysis was restricted to the HCV-positive group.

Determination of serum cytokines. According to the manufacturer's instructions, Visfatin (Phoenix Pharmaceutical, Inc., Burlingame, CA, USA), IL-1 α, IL-1β, IL-10, tumour necrosis factor (TNF)-α (Aushon Biosystems, Billerica, MA, USA), Adiponectin and Insulin (DRG International, Springfield, NJ, USA), Hepatocyte growth factor (HGF) (Aushon Biosystems, Inc., Billerica, MA, USA), and Insulin-like growth factor (IGF)-2 (Boster Biological Technology, Pleasanton, CA, USA), and were measured in duplicate We also tested serum CYFRA21-1 with the human cytokeratin fragment antigen 21-1 (CYFRA21-1) ELISA kit (Cusabio Biotech Co., Ltd., Wuhan, P.R. China) in accordance with the instructions of the manufacturer. Serum IL-6, TNF-α, IL-8, VEGF, Ang1, Ang2, and TGF-β1 levels were determined with the quantikine/high-sensitivity enzyme-linked immunosorbent assay kit (R&D Systems, Minneapolis, MN, USA). Absorbances were measured at 450 and 490 nm using an automatic microplate reader (Multiskan EX; Thermo Fisher Scientific, Inc, Waltham, MA, USA), with background subtraction at 570 and 650 nm, respectively.

Immunohistochemistry. A portion of the formalin-fixed paraffin-embedded liver tissue samples, obtained at enrolment in the study, was used for SIX1's the immunohistochemical evaluation. After deparaffinization and rehydration, antigen unmasking was performed with 1 mM EDTA buffer, pH 8, at 98˚C for 15 mi. Then, these sections were incubated in methanol 5% and H_2O_2 1% for five minutes for blocking endogenous peroxidases, whereas nonspecific sites were blocked using a blocking solution reagent with bovine serum albumin 3% for 30 min at room temperature. Sections were incubated with mouse anti-SIX1 (AB252224; Abcam) primary antibody at a working dilution of 1:60. Thereafter, sections were incubated with prediluted OmniMap anti-mouse horseradish peroxidase-conjugated secondary antibody (Ventana Medical Systems from Tucson, AZ), for 20 min in a humidity chamber as well as with detection kit reagents (ultra-view universal horseradish peroxidase multimer and diaminobenzidine [DAB] chromogen, Ventana Medical Systems) in compliance with the manufacturer's instructions. Subsequently, these sections were counterstained with haematoxylin, dehydrated, and permanently mounted for microscopic examination). To obtain the intensity value of the DAB signal, images of stained liver tissue were processed with ImageJ software (http://rsbweb.nih.gov/.

Determination of oestradiol and testosterone levels. Serum levels of oestradiol and testosterone were determined by ELISA kits (Ella, Bio-Techne, Milan) following the manufacturer's instructions.

MicroRNA analysis. RNAs from the aforementioned cohort of patients were evaluated for miRNA expression and then assayed by quantitative PCR using miRCURY LNA RT Kit (Hilden, Germany). Notably, 10 ng total RNA served as a template for a 10 μl reverse transcription reaction using miRCURY LNA miRNA SYBR Green PCR Kit and miRCURY LNA miRNA PCR Assays (Qiagen). For each miRNA, reactions were done in duplicate on a LightCycler 480 (Roche Applied Science) using the manufacturer's recommendations for cycling parameters. The expression levels of miRNA were measured using the quantification cycle values (Cq values). U6 RNA and RNU5G were taken as controls, and the assays were quantified by the $2^{-\Delta\Delta Cq}$ method (11). We used this method to analyse the relative expression of the miRNAs of interest relative to two endogenous controls and relative to the corresponding cirrhotic non-tumour liver tissue.

Statistical analysis. A comparison between dichotomous and continuous variables was drawn by employing Fisher's exact test, bivariate (Pearson) correlation analysis, and t-test (paired or unpaired), respectively. In case of SIX1 upregulation and survival, the cumulative probability of overall survival was evaluated by the Kaplan-Meier method. Patients were censored at the time of LT, death, or the last available follow-up. Differences in observed probability were assessed using the log-rank test or by two-stage hazard rate comparison method in case of crossing survival curves (12).

The Cox proportional method was utilised for identifying risk factors for the overall survival and growth speed. The variables that underwent testing included: sex, age, E-S grading, presence of macrovascular invasion assessed by CT scan, multifocality at baseline, platelets level, and α-fetoprotein levels. For survival analysis, albumin, creatinine, and bilirubin, were used as additional independent variables. The PASW Statistics 28 program (IBM Corp., Armonk, NY) was used for statistical analysis.

Results

Cohort's description. Seventy-eight patients with liver cirrhosis (Child-Pugh A: n=53; B: n=23; C: n=2) on surveillance for HCC were enrolled in the study reported in (9). Sixty-one (78.2%) of these patients were males. Of these, 10 (12.8%) were Hepatitis B positive, 11 (14.1%) had alcohol-related CLD, and 11 (14.1%) had dysmetabolic CLD. Hepatitis C emerged as the most common aetiology, with 46 (58.9%) patients being HCV-positive [29 male (63.0%) and 17 females (37.0%)]. In females, HCV was the only represented aetiology. We, therefore, focused on the analysis of gender-related aspects of HCV-positive patients.

The HCV-positive cohort's mean age was 68.9±9.2, with males significantly younger than females (M vs. F: 64.0±14.8 vs. 71.0±8.3, P=0.001). Considering the whole cohort of HCV-positive patients, survival was not significantly different between males and females (males vs. females: 36.8±22.7 vs. 39.5±33.2, P=0.705).

Demographic and clinical data of the HCV-positive cohort and the gender-based main HCC characteristics in HCV-positive patients are summarised in Table I. No substantial differences were found in HCC, although a trend towards a higher percentage of E-S grade 3 in females was observed. The significantly higher AFP levels in females at presentation are in line with this finding (Table I).

Global gene expression according to gender. We analysed the original database comprising all genes that had been found differentially expressed in tumour tissue when compared to the non-tumoral cirrhotic tissue for differences in the expression level according to sex. In the entire cohort, we found 198 genes differentially expressed between males and females, irrespective of aetiology. Table SI lists the 133 genes differentially expressed in the HCV-positive cohort. Seventy-six were up regulated in females and 59 in males. Three genes (SIX1, GPR19, ADH1C) were defined by micro-array and high throughput sequencing technologies as possible oestrogen targets (10). Since SIX1 was the only one which was significantly related with both higher HCC growth speed and lower survival in females (see below), we focused our attention on it. SIX1 transcriptomic expression levels, from non-tumour and tumour tissue, stratified based on sex, are shown in Fig. 1A. Levels of expression in non-tumour tissue were not significantly different between males and females while a highly significant, straightforward difference was present in tumour tissue, with females exhibiting the highest levels of expression (P<0.001). The expression level between non-tumour and tumour tissue was significantly different in both males and females (P<0.001). We also evaluated DACH1 expression since many studies suggested that it had a correlation with SIX1 expression (13,14). Although DACH1 expression was not significantly different between males and females, a significant inverse relationship between SIX1 and DACH1 was observed in males, both in the HCV-positive males (males: r=-0.532, P=0.003; females: r=-0.055, P=0.780, Pearson bivariate correlation) and in the entire cohort (r=-0.395, P=0.002, Pearson correlation). The gene TGF-β (14) is also known to interact with SIX1. Upon being upregulated, SIX1 can switch TGF-β signalling to the prometastatic phenotype. In females, we found a notable positive relationship between upregulated SIX1 and TGF-β (r=0.505, P=0.006, Pearson correlation) that was absent in males (r=-.375; P=0.060, Pearson correlation).

Immunohistochemical evaluation of SIX1. Immunohistochemical evaluation of SIX1 in the paired HCC/non-tumour tissue biopsies showed a significantly higher expression in tumoral tissue of female patients when compared to that of males (Figs. 1B and S1). The expression level was not different in nontumoral tissue between males and females while a significant difference was present between SIX1 expression between tumoral and nontumoral liver tissue in females (Fig. 1B).

Correlations between SIX1 expression and pathologic features. A distinctive pattern of relationships was found between sex, pathologic features, and SIX1 expression. E-S grading only was positively correlated with increased SIX1 expression in HCV-positive males while neither inflammatory intensity, maximal nodule size, Ki67, e-cadherin gene nor protein expression in HCC had such a correlation. On the contrary, inflammatory intensity, e-cadherin gene and protein expression were significantly correlated in HCV-positive

Table I. Demographic and clinical characteristics (including those related to HCC) of HCV-positive patients at presentation according to sex.

Variable	Whole cohort (n=46)	Males (n=29)	Females (n=17)	P-value
Age, years	68.9±9.2	64.0±14.8	71.0±8.3	0.001
Child-Pugh score				0.549
A	38	23	15	
B	8	6	2	
MELD	11.4±3.2	11.6±3.2	10.6±3.2	0.264
AFP, ng/ml	122±596	109±387	1.949±6.931	0.049
Bilirubin, mg%	1.8±3.1	1.9±3.2	1.6±2.6	0.810
Albumin, g/l	3.6±0.56	3.5±0.59	3.6±0.44	0.533
Creatinine, mg/dl	0.95±0.36	1.0±0.33	0.771±0.24	0.019
INR	1.30±0.18	1.31±0.18	1.29±0.19	0.639
Platelets, $x10^3/mm^3$	114±61	112±61	120±58	0.655
HCC doubling time, days	109±100	114±111	102±81	0.702
Tumour volume, log cm^3	3.8±0.9	3.7±1.1	3.9±7.2	0.613
Multifocality at baseline (%)	7 (15.2)	6 (20.7)	1 (5.9)	0.391
AFP >400 ng/ml (%)	3 (6.5)	0	3 (17.6)	0.525
Macrovascular invasion, n (%)	3 (6.5)	2 (6.9)	1 (5.9)	0.558
BCLC class (%)				0.653
A	32 (69.6)	19 (65.5)	13 (76.5)	
B	9 (19.6)	6 (20.7)	3 (17.6)	
C	5 (10.9)	4 (13.8)	1 (5.9)	
Edmondson-Steiner grade (%)				0.086
1	16 (34.8)	11 (37.9)	5 (29.4)	
2	17 (37.0)	13 (44.8)	4 (23.5)	
3	13 (28.3)	5 (17.2)	8 (47.1)	
Treatment (%)				0.743
Supportive care	13 (28.3)	6 (20.7)	7 (41.2)	
Liver transplant	1 (2.2)	1 (3.4)	0	
Resection	2 (4.3)	1 (3.4)	1 (5.9)	
TACE	17 (36.9)	11 (37.9)	6 (35.3)	
RFA	10 (21.7)	8 (27.6)	2 (11.8)	
Sorafenib	2 (4.3)	1 (3.4)	1 (5.9)	
Sequential treatments	1 (2.2)	1 (3.4)	0	

Data are presented as counts or as mean ± SD. MELD, Model of End-stage Liver Disease; INR, International Normalized Ratio; AFP, alphafetoprotein; BCLC, Barcelona Clinic Liver Cancer; TACE, transarterial-chemoembolization; RFA, radiofrequency ablation.

females, with e-cadherin having an inverse relationship with SIX1.

On the contrary, inflammatory intensity, e-cadherin gene, and protein expression were significantly correlated in HCV-positive females, with e-cadherin having an inverse relationship with SIX1.

SIX1 transcriptomic upregulation relates with higher growth speed in females. A higher SIX1 expression had a strong relationship with the probability of having a higher growth speed both in the group of HCV-positive patients (P=0.007, log-rank test) and the entire cohort of patients (P=0.014, log-rank test). However, in the former group, stratification by gender showed that the probability of having a higher growth speed was highly significant in females (P=0.009, log-rank test), but was absent in males (P=0.228, log-rank test) (Fig. 2A and B).

HCC doubling time in the six weeks after diagnosis was significantly inversely related to SIX1 expression levels in females (r=-0.575, P=0.001, Pearson correlation). No significant relationship was found in males (r=-0.309, P=0.103, Pearson correlation). The transcriptomic signature was related in the initial report to survival as well as growth speed (9). Three of the individual components of the transcriptomic signature (ANGPT2, DLL4, ESM1, NETO2, NR4A1) were differentially related with SIX1 expression in the HCV-positive cohort as a whole: ANGPT2 (r=0.292, P=0.026, Pearson correlation); DLL4 (r=0.344, P=0.008, Pearson correlation); ESM1 (r=0.459; P<0.001, Pearson correlation). Stratifications

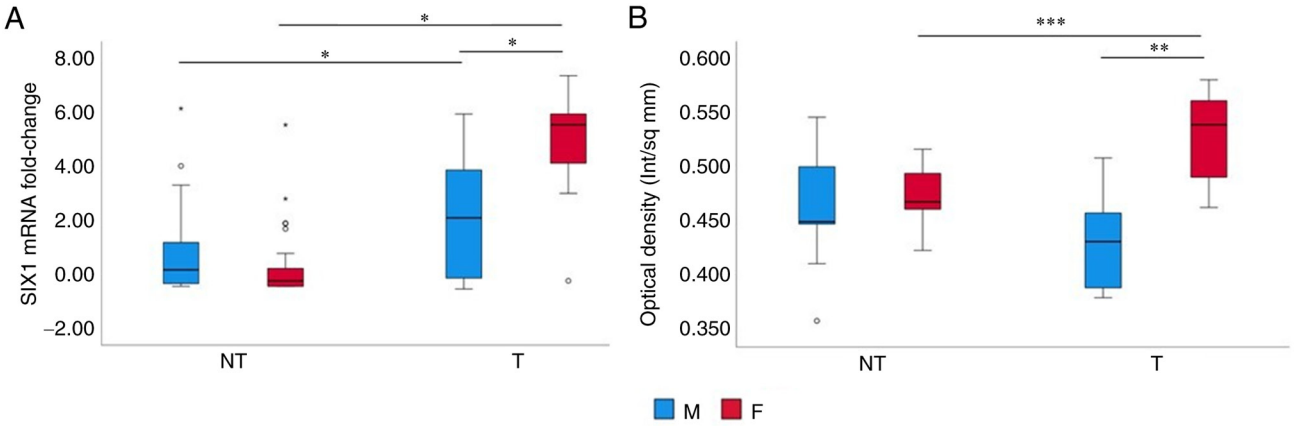

Figure 1. Transcriptomic and proteomic levels of SIX1 in nontumor and tumour tissue according to sex. (A) mRNA levels were evaluated by microarray analysis (Agilent Whole Human Genome Oligo Microarrays). Expression levels in non-tumor tissue were non-significantly different between males and females. In the tumour tissue, SIX1 levels only moderately increased in males while in females a sharp increase was observed. Comparisons were performed by paired t-test between T and NT in males, and T and NT in females. T-test for independent groups was used to compared SIX1 in tumour tissue of males vs. females, as well as in non-tumor tissue of males vs. females. (*P<0.001)Graphs were organized as presented to make visual comparison easier but statistical analysis performed between indicated pairs. All significant comparison are indicated by horizontal bars. (B) Immunohistochemical evaluation of SIX1 in paired HCC/non-tumoral liver biopsy, representative images are presented in Fig. S1. Similar levels of expression were present between males and females in nontumor liver tissue while in HCC tissue significantly higher levels were present in females vs. HCC in males. Tumoral SIX1 levels in females were also significantly higher vs. their respective nontumor tissue (**P=0.002 and ***P=0.005; t-test for independent groups). Bars indicate the groups compared and asterisks the level of significance. Comparison between T and NT tissue within male and female group was made by paired t test. NT, non-tumour; T, tumour; M, male; F, female.

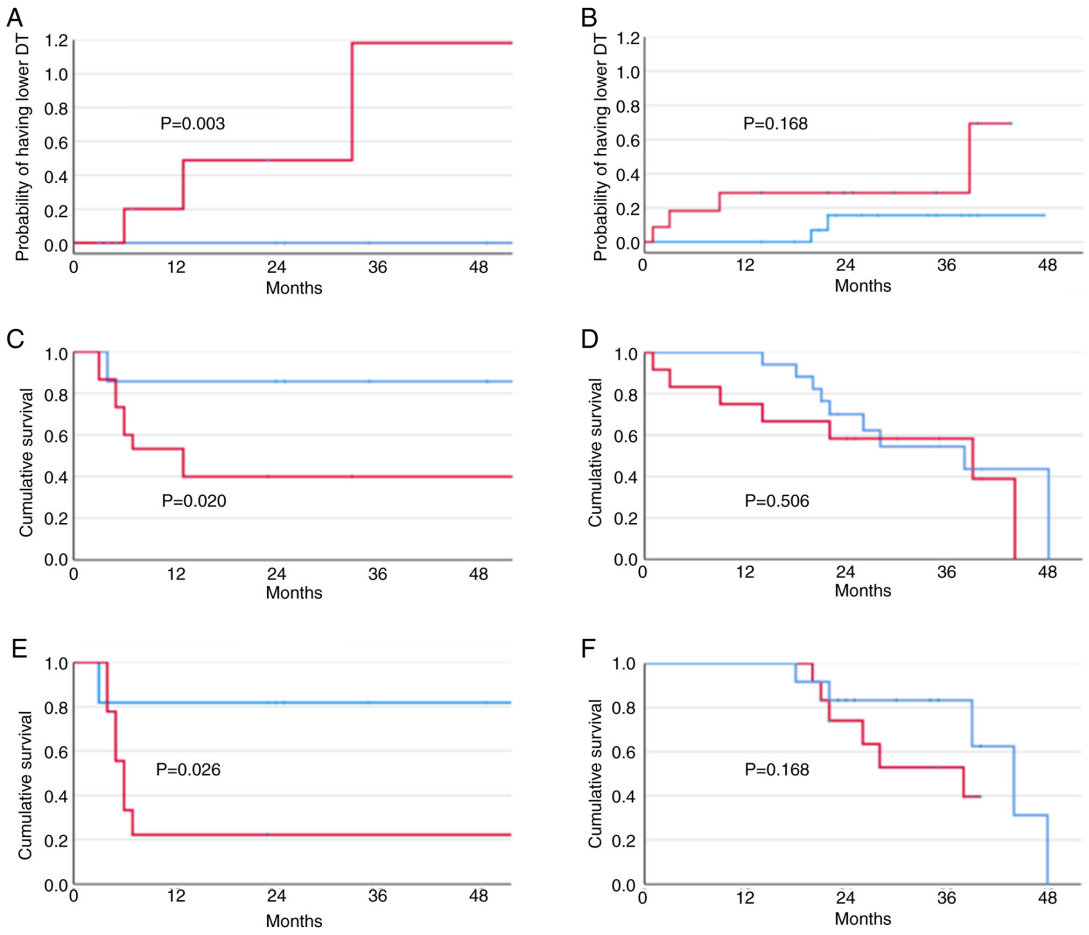

Figure 2. Evaluation of growth speed (evaluated by the estimate of the doubling time) and of survival in HCV-positive females and males in relation to low or high SIX1 expression. (A and B) Probability of having elevated growth speed in relation to SIX1 median values in (A) females and (B) males. HCC in females who had elevated SIX1 median values was characterised by significantly higher growth speed (P=0.003 by log-rank test). (C) HCV-positive females with higher median SIX1 levels had significantly lower survival in comparison with those with lower median levels (P=0.020, log-rank test). (D) No difference was present in males in relation to SIX1 levels. (E and F) Survival analysis was performed according to SIX1 proteomic expression in the tumour tissue of (E) females and (F) males. Data confirm survival analysis according to transcriptomic data. The probability of having elevated speed growth and survival was evaluated by the Kaplan-Meier method. For panel 2C and 2E two-stage hazard rate comparison method was used. DT, doubling time.

by sex displayed that in females, SIX1 had a significant relationship with ANGPT2 (r=0.378, P=0.043, Pearson correlation); NETO2 (r=0.612, P<0.001, Pearson correlation); and ESM1 (r=0.376, P=0.044, Pearson correlation) while a significant relationship was present in males with DLL4 (r=0.499, P=0.015, Pearson correlation), and ESM1 (r=0.496; P=0.006, Pearson correlation). Quantitative analysis of the relationship between median SIX1 values and dichotomic value of the transcriptomic score (bland vs. aggressive) showed that all seven women with lower median SIX1 levels had a bland HCC while four of them with higher median values had an aggressive HCC (P=0.029). No difference was found in males between those with higher and lower SIX1 median levels (P=0.158).

Factors predictive of growth speed at Cox regression analysis. Of the variables tested (sex, age, E-S grading, presence of macrovascular invasion at CT scan, multifocality at baseline, platelet level, α-fetoprotein levels, SIX1 levels), only the presence of macrovascular invasion at CT scan (HR: 6.670, 95% CI 1.689 to 4.505, P=0.007), E-S grading (HR: 3-909, 95% CI 1.371 to 11.143, P=0.011), multifocality at baseline (HR: 2.761, 95% CI 1.692 to 6.382, P<0.001), and SIX1 levels (HR: 6.024, 95% CI 1.314 to 27.622, P=0.021) were predictive of growth speed at univariate Cox regression analysis. As all the significant factors were collinear, multivariable analysis was not performed. Analysis of the Cox results for the different variables based on sex revealed that only multifocality at baseline was significant in both males and females while there was a highly significant relationship in females only for the presence of macrovascular invasion at CT scan and SIX1 (females: macrovascular invasion: HR: 9.721, 95% CI 1.367 to 69.111, P=0.023; SIX1: HR: 2.803, 95% CI 1.132 to 6.382, P=0.026; males: macrovascular invasion: HR: 3.097, 95% CI 0.343 to 28.000, P=0.314; SIX1: HR: 1.215, 95% CI 0.098 to 1.645, P=0.207).

SIX1 upregulation relates with lower survival in females. No significant difference was observed in the entire HCV-positive population regarding survival in relation to SIX1 upregulation (P=0.119, log-rank test). However, stratifying the HCV-positive cohort by sex, a significantly lower survival was present in HCV-positive females (P=0.020, log-rank test). On the other hand, no difference was found in males (P=0.772, log-rank test) (Fig. 2C and D). The same finding was obtained after stratifying survival by median histochemical SIX1 values (Fig. 2E and F).

Factors predictive of survival at Cox regression analysis. Of the variables examined at univariate analysis (sex, age, E-S grading, presence of macrovascular invasion assessed by CT scan, multifocality at baseline, the presence of ascites, encephalopathy, platelet level, α-fetoprotein levels, albumin, creatinine, and bilirubin) in the whole HCV-positive cohort, only the number of nodules at entry into the study HR: 1.840, 95% CI 1.363 to 2.484, P=0.001), albumin levels (HR: 0.335, 95% CI 0.158 to 0.712, P=0.004), and SIX1 upregulation (HR: 2.288, 95% CI 1.058 to 4.947, P=0.035) were found to have a significant relationship with survival. Multivariable analysis was prevented by the collinearity between the three significant factors. Analysis after stratification by sex revealed that for both males and females, the number of nodules at entry was significantly related with survival (women: HR 1.791, 95% confidence interval, 1.160 to 2.765, P=0.009, men: HR 1.715, 95% confidence interval, 1.127 to 2.609, P=0.012). Albumin and SIX1 upregulation were significant for females only (SIX1: women HR 5.034, 95% CI 1.083 to 23.387, P=0.039, men: HR 1.407, 95% confidence interval, 0.508 to 3.895, P=0.511; albumin: women HR 0.086, 95% confidence interval, 0.019 to 0.398, P=0.002, men: HR 0.610, 95% confidence interval, 0.196 to 1.899, P=0.394).

Table II. Correlation between SIX1 and miRNA expression in HCV-positive males and females.

MicroRNA	Males	P-value	Females	P-value
miR-421	0.431	0.036	0.162	0.461
miR-9-5p	0.429	0.041	0.478	0.033
miR-181b	-0.136	0.528	0.578	0.006
miR-503-5p	-0.021	0.924	0.626	0.002
miR-125b	-0.251	0.248	-0.437	0.037
miR-19b-1-5p	0.258	0.235	0.509	0.018
miR-139-5p	-0.084	0.703	-0.438	0.037
let-7c-3p	-0.011	0.963	-0.536	0.027
let-7c-5p	-0.065	0.762	-0.440	0.035
miR-26b	-0.131	0.543	-0.465	0.025
miR-1303	0.257	0.226	-0.409	0.053

Relationship between SIX1 upregulation and miRNA. In the entire HCV-positive cohort, only miR-421, miR-9-5p, and miR-19b-1-5p were found to have a significant relationship with SIX1 upregulation. However, when the HCV-positive cohort was stratified by sex, a very distinctive and different miRNA pattern was present in relation to SIX1. In males, only miR-421 and miR-9-5p were related with SIX1. In females, several more miRNAs were found to be related with SIX1: miR-181b, miR-503-5p, and miR-125b had a direct relationship, while miR139-5p, miR-26b, let7c-3p, and let7c-5p had an inverse relationship (Tables II and III).

Oestradiol and testosterone levels. No significant difference was present between males and females in the level of circulating oestradiol or testosterone (Fig. S2). However, a significantly lower concentration of circulating oestradiol was found in those overexpressing SIX1 (P<0.001 when stratifying by SIX1 median levels, within the female group), while testosterone had higher, although only borderline significant, levels (P=0.069). Females with HCCs overexpressing SIX1 were found to have significantly higher TGF-β1 and HGF levels and lower Visfatin levels.

Of the large panel of 17 cytokines tested, very different results were found in males and females in relation to SIX1 expression. In females TGF-β1 (r=0.428, P=0.029, Pearson correlation) and HGF (r=0.639, P<0.001, Pearson correlation) were positively correlated with SIX1 levels while Visfatin (r=-0.599, P=0.002 Pearson correlation) was inversely related. A positive relationship between both TGF-β1 and SIX1 was

Table III. Relationship between median SIX1 expression values and level of expression of the significantly associated miRNA at Pearson bivariate correlation.

		Six1		
miRNA	Median miRNA value	Lower median value	Higher median value	P-value
Males				
miR-421	0.380	Downregulated	Normally regulated	0.009
miR-9-5p	0.135	Downregulated	Normally regulated	0.049
Females				
miR-9-5p	0.135	Downregulated	Normally regulated	0.025
miR-181b	-0.340	Downregulated	Upregulated	0.003
miR-503-5p	-0.780	Normally regulated	Highly downregulated	0.004
miR-125b	-0.880	Downregulated	Extremely downregulated	0.037
miR-19b-1-5p	0.654	Downregulated	Upregulated	0.001
miR-26b	-0.980	Normally regulated	Normally regulated	0.025
let7c-3p	-0.650	Upregulated	Downregulated	0.017
let7c-5p	-0.310	Upregulated	Downregulated	0.017
miR-139-5p	-0.910	Normally regulated	Extremely downregulated	0.037

found within AFP (r=0.595, P=0.001, and r=0.378, P=0.048, respectively, Pearson correlation).

In males, none of the cytokines tested but HGF was found to have a positive correlation with SIX1 levels (r=0.462, P=0.017 Pearson correlation). Visfatin levels inversely related with BMI (r=-0.573, P<0.001 Pearson correlation) in females but not in males were.

Discussion

In this study, we have shown that addressing the onset and the course of HCC without stratifying by sex can be grossly misleading. Evaluation of the HCC data keeping female and male patients together would have caused us to miss very distinctive features that became obvious during a separate sex analysis. We performed a detailed evaluation of gene expression in males and females from a very well-characterised cohort of HCC patients at first HCC diagnosis, restricting the analysis to the HCV-positive subgroup, as no females with other aetiologies were represented in the whole cohort (9). After evaluating which genes were differentially expressed between males and females in the entire cohort, we then assessed those that were differentially expressed in the HCV-positive cohort and which, of these, were possible targets of oestrogen action. Accordingly, we identified three differentially expressed genes (SIX1, GPR19, ADH1C), which are reported as possible oestrogen targets (10). We focused our attention on SIX1 as it was the only one, of the three indicated above, that was significantly associated with growth speed and survival in females.

According to SIX1 expression (which was also confirmed at the proteomic level), analysis of the relationship of males and females with pathologic features showed that the only significant association in males was with E-S grading. None of the other pathologic features considered (inflammatory activity, e-cadherin expression) was significantly related. In females, no relationship was found between upregulated SIX1 and E-S grading while other features such as increased inflammatory activity and decreased e-cadherin expression (both at RNA and at protein level) were significantly associated. This last feature is particularly interesting given the relationship we found, in females only, between SIX1 upregulation and TGF-β up regulation in tumour tissue and increased levels in serum. In this regard, Micalizzi *et al* (15,16) demonstrated in breast cancer that while TGF-β upregulation is sufficient to induce epithelial-mesenchymal transition (EMT), SIX1 upregulation is required to determine the switch of TGF-β signalling to the prometastatic phenotype. Meanwhile, Min and Wei (17) demonstrated that silencing SIX1 was able to inhibit TGF-β/Smad2/3 pathway, suppressing EMT. Liu *et al* (18) hand showed that SIX1 enhances the TGF-β signalling pathway by upregulating TGFβ-R2 expression and that deletion of Six1 in cancer cells significantly reduced tumour growth in an immune-dependent manner with enhanced antitumor immunity in the TME. The addition of SIX1 upregulation is a further piece of knowledge to the complex microenvironment that we already described for TS-positive aggressive HCC, i.e. marked PD-1 and PD-L1 upregulation, prominent EMT, and clear-cut activation of TGFβ1 signalling (19). In this subgroup of HCV-patients females with aggressive HCC, we found a positive relationship between higher circulating TGFβ levels and upregulated SIX1 as well as a positive relationship between them and significantly higher AFP levels. In addition, several genes composing the transcriptomic signature (ANGPT2, NETO2, ESM1) were also upregulated and had a positive relationship with SIX1 upregulation in females HCC, which were also characterised by higher growth speed and lower survival. All these features point toward an increased biologic aggressiveness for HCC overexpressing SIX1 in females.

The SIX1 gene encodes a homeodomain-containing transcription belonging to the 6th family of homeoproteins. SIX1 was found to be linked to the development of tissues and organs, thus potentially promoting the proliferation and survival of

precursor cells before cell differentiation (20). An important role in cell apoptosis has also been reported (21,22). In human cancer, elevated levels of SIX1 mRNA were found in early and late-stage ovarian cancer (21). SIX1 upregulation has been also associated with poor prognosis in breast cancer (23), in gastric cancer (24), and in colorectal cancer (25). More recently, a few studies have reported that SIX1 has a relevant role in HCC as well: SIX1 upregulation was identified as an independent poor prognostic factor of HCC (26,27). Cheng *et al* (22) demonstrated that SIX1 upregulation was linked to tumorigenesis and that its suppression, coupled with induction of DACH1 upregulation, inhibited the progression of HCC both *in vitro* and *in vivo*. In our series, a significant relationship between SIX1 and DACH1 was present in the whole HCV-positive cohort and in the HCV-positive males but not in females. This could be indicative of the fact that the mechanisms proposed by the authors, i.e. suppression of tumorigenesis via p53 up-regulation, via SIX1 inhibition, and DACH1 upregulation, might be valid for males only while in females SIX1 could act via other mechanisms. Accordingly, a contrasting gene expression pattern comparable to our results was shown in male and female breast cancer (28). In this study, DACH1 and SIX1 had contrasting expression pattern in males and females and comparable opposite prognostic implication. Similar to other findings, females with breast cancer overexpressing SIX1 had more aggressive disease and severe prognosis.

SIX1 is known to directly interact with oestrogens (10). Its role in hormonal carcinogenesis has been demonstrated in experimental as well as human endometrial carcinogenesis (29). Neonatal exposure to phytoestrogens or diethylstilbestrol could be followed in later life by aberrant endometrial expression of SIX1 and eventually by endometrial carcinoma (30,31). It is certainly difficult to ascertain whether this group of HCV-positive women had had any early hormonal exposure; it is more likely that if present, had occurred later in life. On the other hand, no comparable data are available for an organ, like the liver, which is a non-classical target for oestrogen. The presence of α-oestrogen receptors in the liver functionally identical to those of the classical target organs (32) offers the potential physiologic basis for similar mechanisms to occur. Interestingly, women overexpressing SIX1 were found to have significantly lower circulating concentrations of oestradiol and higher levels of testosterone (although the latter did not reach full significance), despite all having an age indicating advanced menopause. This hormonal framework has already been elucidated in menopausal HCV-positive women with advanced fibrosis and resistance to antiviral therapy, thereby suggesting that this modification during the course of chronic liver disease can contribute to the loss of anti-inflammatory action linked with oestrogens, aggravated by the contemporary increase of androgens hormones (33).

The pattern of the activated miRNA in association with SIX1 was also very distinctive between males and females. While we found only miR-421 and miR-9-5p positively upregulated in males, a much larger number was found in females. Some upregulated (miR-181b, miR-19b_1_5p) whereas most of them were down-regulated (miR-139-5p, miR-503-5p, miR-125b miR-26b, let7c-3p, let7c-5p), all of them in association with SIX1 upregulation. The presence of this relevant number of upregulated or downregulated miRNA was not evident when the cohort was examined as a whole, probably because the lower number of females in respect to males did not allow the revelation of their specific patterns. These different miRNAs combinations found exclusively in females are quite informative. Meng *et al* (34) have already described the combination of upregulated miR-181b and downregulated let7 and suggested that it could represent a molecular target in HCC and a possible therapeutic tool for eradication of hepatocellular malignancies. A possible functional role as an oncogene has been suggested for miR-181b (35). Zhou *et al* (36) showed that miRNA-181b was significantly upregulated in response to TGF-β treatment in gastric cancer cell lines via induction of Smad2/3 signalling. Interestingly, SIX1 is known to activate the TGF-β/Smad2/3 pathway, and silencing SIX1 blocks EMT via inhibition of TGF-β/Smad2/3 signals (17). Similarly, downregulated miR-503-5p, as we have found in HCV-positive females in association with upregulated SIX1, has been linked to increased EMT (37) and increased HCC progression (38). By contrast, upregulated mir503 inhibits cellular proliferation and induces apoptosis in HCC cells (39) and can sensitize HCC cells to 5-fluorouracil (40). Concordantly, the downregulation of miR-139-5p (41) and of miR-125b (42) was associated with increased EMT and increased metastatic capacity. A recent study in patients with HCC revealed that the downregulation of miR-139-5p resulted in poor survival (43). In its entirety, the peculiar miRNA pattern evidenced in females HCC overexpressing SIX1 points towards a specific activation of pathways associated with relevant biologic aggressiveness of the tumour, a feature that is concordant with the clinical course of these patients.

The novelty of our findings is the association with SIX1 upregulation in females, a relationship that was yet to be explored previously. However, there are some limitations in his study: one resides in the lack of an experimental demonstration of the ability of SIX1 upregulation to modify hepatic cellular reactivity toward a higher oncogenic ability. Nevertheless, our findings seem strong enough to suggest the opportunity to conduct an experimental exploration of the effect of SIX1 upregulation in liver carcinogenesis in females. Secondly, an explanation for the specific activation of SIX1 in females should be sought. A starting point to explore could be represented by a careful epidemiologic and anamnestic study in women with HCC to discover possible hormonal exposure that could, as in the case of endometrial cancer, offer a key to the interpretation of this selective upregulation.

Overall, on the one hand, these data suggest a very distinctive model for carcinogenesis, unique to HCV-positive women, characterised by a marked downregulation of potentially protective mechanisms against excess proliferation, EMT, and metastatic capacity, and by a marked activation of potential oncogenes on the other hand. All these mechanisms are in relation to a gene, SIX1, which has a close relationship with estrogenic control. However, it is not completely clear and deserves a further evaluation of how this gene can influence liver carcinogenesis specifically in females.

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Availability of data and materials

Gene expression data are available at the Gene Expression Omnibus website (http://www.ncbi.nlm.nih.gov/geo) under the accession number: GSE54236. The other datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

RMC, FM, SL, AR, SM and FD and GM performed experiments, and analysed and interpreted experimental data. FS, DR, AP and LDM identified and followed up with suitable patients and compiled and analysed the clinical database. RMC, FM, MLMC, GG and EV interpreted the results. RMC, FM, FS, MLMC and GG reviewed the manuscript. RMC, FM, FS, DR, MLMC and GG approved the final version of the manuscript. GG and EV confirm the authenticity of all the raw data. EV conceived and designed the study and wrote the paper. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The study was conducted according to the Declaration of Helsinki and approved by or Ethics Committee of University Hospital of Modena (IRB10/08_CE_UniRer; ClinicalTrials ID: NCT01657695). Written informed consent was obtained from all subjects involved in the study.

Patient consent for publication

Written informed consent included consent to publication.

Competing interests

The authors declare that they have no competing interests.

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