Dynamic expression of H19 and MALAT1 and their correlation with tumor progression biomarkers in a multistage hepatocarcinogenesis model

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Abstract
Hepatocellular carcinoma (HCC) progresses sequentially in a stepwise pattern. Long noncoding RNA (IncRNA) can regulate the complex cascade of hepatocarcinogenesis. Our study aimed to elucidate the expression profile of H19 and MALAT1 during the different stages of hepatocarcinogenesis and the correlation between H19 and MALAT1 with the genes implicated in the carcinogenesis cascade. We employed a chemically induced hepatocarcinogenesis murine model to mimic the successive stages of human HCC development. Using real-time PCR, we analyzed the expression patterns of H19 and MALAT1, as well as the expression of biomarkers implicated in the Epithelial-Mesenchymal transition (EMT). The protein expression of the mesenchymal marker vimentin was also evaluated using immunohistochemistry in the stepwise induced stages. The histopathological evaluation of the liver tissue sections revealed significant changes during the experiment, with HCC developing at the final stage. Throughout the stages, there was a dynamic significant increase in the expression of H19 and MALAT1 compared to the normal control. Nevertheless, there was no significant difference between each stage and the preceding one. The tumor progression biomarkers (Matrix Metalloproteinases, vimentin, and β-catenin) exhibited the same trend of steadily increasing levels. However, in the case of Zinc finger E-box-binding homeobox 1 and 2 (ZEB1 and ZEB2), the significant elevation was only detected at the last stage of induction. The correlation between IncRNAs and the tumor progression biomarkers revealed a strong positive correlation between the expression pattern of H19 and MALAT1 with Matrix Metalloproteinases 2 and 9 and vimentin. Our findings imply that genetic and epigenetic alterations influence HCC development in a stepwise progressive pattern.

KEYWORDS
diethylnitrosamine, epithelial-mesenchymal transition, hepatocellular carcinoma, lncRNA, matrix metalloproteinases, noncoding RNA
1 | INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the world’s leading causes of cancer mortality as it is a very aggressive solid malignancy. Annually, the incidence of HCC is increasing dramatically. Developing novel therapeutic strategies requires a thorough understanding of the underlying molecular pathways contributing to HCC development and progression. Hepatocarcinogenesis involves a complex interplay of various signaling pathways. Among them is the Epithelial-Mesenchymal transition responsible for poor prognosis. Trans-differentiation of the non-motile polarized epithelial cells into motile nonpolarized mesenchymal cells is required for tumor cell growth and contributes pathologically to fibrosis and cancer progression. Several essential transcription factors, including the SNAIL superfamily and members of the zinc-finger E-box-binding homeobox (ZEB1 and ZEB2), are involved in this transition in cell differentiation and behavior. ZEB family members function by suppressing E-cadherin, the cell adhesion molecule, thus inducing EMT. Elevated levels of ZEB1 and ZEB2 have been linked to tumor progression and poor overall survival in patients with hepatocellular carcinoma and gastric cancer. Matrix metalloproteinases (MMPs) are a class of zinc-dependent endo-proteases that degrade different proteins in the extracellular matrix (ECM) and hence participate in essential cellular processes such as proliferation, differentiation, adhesion, and apoptosis. MMP family members, such as MMP2 and MMP9, play critical roles in the complex pathogenesis of EMT during liver carcinogenesis. MMP members are engaged in different levels of liver conditions (i.e., injury, inflammation, fibrosis, cirrhosis, and hepatocarcinogenesis) with varying expression levels. Therefore, they are recognized as biomarkers for diagnosing and staging liver diseases. Wnt/β-catenin signaling is another tumor-promoting pathway that is involved in hepatocarcinogenesis. The aberrant Wnt/β-catenin signaling, through the activation of β-catenin, promotes the development and progression of liver cancer.

Noncoding RNAs (ncRNAs) have recently received more attention owing to their crucial biological implications in cancer development and progression. ncRNAs include microRNAs, circular RNAs, intronic RNAs, and long noncoding RNAs (lncRNAs). Many studies focused on the functional roles of miRNAs as diagnostic and prognostic biomarkers for various cancer types. However, recent studies highlighted the critical importance of lncRNAs in regulating gene expression at the transcriptional or post-transcriptional level. Numerous lncRNAs have been identified as oncogenes or tumor suppressors, with implications in cell proliferation, EMT, and angiogenesis, in addition to other cellular processes. IncRNAs are promising tumor diagnostic and prognostic indicators owing to their cancer-specific expression and detectable abundance in various clinical samples such as blood and urine. Therefore, a better understanding of HCC-specific IncRNAs will help immensely in HCC diagnosis and treatment.

The first IncRNA identified with a regulatory role in cancer was Metastasis-Associated Lung Adenocarcinoma Transcript 1 (MALAT1). MALAT1 regulates several liver diseases, including hepatic fibrosis, liver regeneration, fatty liver condition, and liver cancer. MALAT1 overexpression has been associated with disease progression, poor clinical outcomes, short overall survival, and the risk of recurrence in patients with liver cancer. As a result, MALAT1 expression may be employed as a cancer biomarker and is considered a hepatocarcinogenesis-associated factor. The oncogenic long ncRNAs H19 is paternally imprinted and postnatally repressed at birth in most tissues. H19 is re-expressed in various cancer types, including HCC. The significance of H19 in the pathogenesis of HCC is controversial. Several studies demonstrated that H19 has a tumor-suppressive effect on the development of HCC. But on the other hand, other studies suggest that H19 could have an oncogenic function.

Previous studies focused on investigating the lncRNA signatures associated with HCC. However, we were interested in assessing the dynamic expression of H19 and MALAT1 in a hepatocarcinogenesis model in which tumor development occurs stepwise and is preceded by chronic inflammation, as it typically occurs in human cases. Collecting serial clinical samples that depict the different stages of liver tumors is difficult. Nevertheless, the various stages of liver disease developed using the Diethylnitrosamine-(DEN)-induced hepatocarcinogenesis rat model can be an efficient and applicable tool to study the underlying molecular mechanism involved in the stepwise stages of tumor development. The two-stage chemical model using an initiator and promoter could produce a stepwise histopathological and genetic progression similar to humans. In the present study, we induced a stepwise hepatocarcinogenesis murine model through the two-stage chemical model using DEN and Nitrosomorpholine (NMOR).

This study aimed to examine the dynamic expression of the lncRNAs H19 and MALAT1 during the stepwise hepatocarcinogenesis process using a murine model miming the multistage progression of liver cancer. We also evaluated the association of H19 and MALAT1 with the expression of several tumor-progression genes that regulate liver tumorigenesis.
2 MATERIALS AND METHODS

2.1 Experimental animals

Male Sprague Dawley rats ($n=80$), 5 to 6-week-old, with a body weight of 120–140 g, were purchased from the Animal Facility Unit at the National Research Centre, Egypt. Animals were caged in 6 groups and maintained at a temperature of 27°C with a 12 h light/dark cycle. The rats were provided with commercially pelleted rat chow and water ad libitum. The experiment conformed to the guidelines for the Care and Use of Laboratory Animals from the Ethical Committee of the National Research Centre. The ethical committee evaluated and approved the animal study protocol. The animals were acclimated for 2 weeks before the start of the tumor induction.

2.2 Induction of the stepwise liver carcinogenesis model

The animal model of DEN-NMOR-induced liver carcinogenesis is developed following the protocol of Futakuchi et al. with some modifications. A total of 80 rats were randomly divided into 5 intervention groups (12 rats/group) representing the different stages of progression. The sixth group considered as normal control, consisting of 20 rats, was in parallel with the other groups during the induction period and only received drinking water. The starting point of the animal experiment was defined as week 1, where the five intervention groups were intraperitoneally injected with DEN at a dose of 100 mg/kg body weight. Then 1 week following the DEN injection, rats received 80 ppm NMOR in the drinking water for 3 weeks. The group was killed at the end of the 4th week, representing a stage in the induction process. This cycle was repeated four times for a total of five cycles (representing five stages), where DEN was injected at the beginning of the 1st, 5th, 9th, 13th, and 17th week followed by 2 weeks of NMOR in the drinking water and then scarifying a group by the end of the 4th, 8th, 12th, 16th, and 20th week (Figure 1). The parallel normal control group, where mice only received distilled water, was collected with the last intervention group at the final collection time.

Liver organs were removed and washed in cold PBS for each collected group. A portion of the tissue was preserved in 10% formaldehyde for histological examination. The remaining tissue was divided into two parts. For biochemical analysis, one part was weighed and homogenized in a precool lysis buffer with an electrical homogenizer. The other part was submerged in RNALater immediately and kept at −80°C for RNA extraction.

FIGURE 1 Diagrammatic scheme of the multistage chemical-induced HCC model applied in the present study. The DEN was intraperitoneally injected at a dose of $100 \, mg/kg$ body weight at weeks 1, 5, 9, 13, 17 representing the first week of the month. Rats received 80 ppm NMOR in the drinking water for 3 weeks following DEN injection. A group is collected at the end of each month (DEN-NMOR cycle). Each group collection’s time point is considered a stage in the induction process. The experiment lasted 5 months, with a final output of five induced groups and one control group in parallel. DEN, Diethylnitrosamine; HCC, hepatic cell carcinomas; NMOR, N-nitrosomorpholine.
2.3 | Histopathological examination

The liver tissue sections were embedded in paraffin blocks and sectioned into 5 μm thick slices on slides for hematoxylin and eosin staining, followed by microscopic analysis to detect histological abnormalities at each stage of the stepwise carcinogenesis process.

2.4 | RNA extraction and reverse transcription

Liver tissue sections stored in RNA later were homogenized using TissueLyser II (Qiagen GmbH) for a sufficient disruption and homogenization through the grinding effect of stainless-steel beads 5 mm (Qiagen, Cat# 69989). Total RNA was extracted from homogenized samples using miRNeasy RNA isolation kit (Qiagen, Cat# 217084) following the instructions provided in the kit. The concentration and purity of extracted RNA were evaluated using a Nanodrop spectrophotometer. The concentration of tissue RNA varied from 900 to 2000 ng/μL. Directly after the isolation, RNA was reverse-transcribed.

For InRNA expression analysis, RT² First Strand Kit (Qiagen, Cat#330404) was used for reverse transcription. Following the manufacturer’s procedure, we used 1 μg of extracted RNA. A genomic DNA elimination step was first conducted, followed by adding the reverse transcription mix and incubating the total mixture at 42°C for 15 min, then immediately stopping the reaction at 95°C for 5 min.

For mRNA expression analysis, the RevertAid First Strand cDNA synthesis kit (ThermoFischer Scientific, Cat#K1622) was used for reverse transcription. Following the manufacturer’s procedure, we used 1 μg of extracted RNA. A genomic DNA elimination step was first conducted, followed by adding the reverse transcription mix and incubating the total mixture at 42°C for 15 min, then immediately stopping the reaction at 95°C for 5 min.

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2.5 | Expression analysis of IncRNAs H19 and MALAT1

Assessment of the aberrant expression of H19 and MALAT1 was conducted by quantitative real-time PCR (RT-qPCR) using RT²-qPCR primer assays for rat H19 (#LPR07420A, Qiagen collection) and rat MALAT1 (#LPR08519A, Qiagen collection). The RT-PCR reaction was performed using RT² SYBR Green master mix (Cat#330520, Qiagen GmbH) on QuantStudio™5 Real-Time PCR System (Applied Biosystems, Thermo Fischer Scientific). The cycling conditions were set to 95°C for 10 min as a holding stage, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. Ct values of H19 and MALAT1 were normalized to the internal reference GAPDH-ps1 (#LPR07395A, Qiagen collection).

2.6 | Expression analysis of ZEB1, ZEB2, β-catenin, and vimentin

Evaluating the mRNA expression of the genes involved in tumor progression was conducted by RT-qPCR on QuantStudio™5 Real-Time PCR System using GoTaq® qPCR master mix (Promega, Cat# A6010). Primers used were designed by PrimerQuest™ Tool (Table 1). The cycling conditions applied were as follows; 95°C for 3 min, followed by 40 cycles of 95°C for 15 s, 60°C for 1 min. The Ct values of the investigated genes were normalized to β-actin. Data were calculated by the 2^ΔΔCt method and represented as relative expression (fold change).

2.7 | Evaluating the levels of active MMP2 and MMP9

MMP2 and MMP9 are synthesized as inactive zymogens and then enzymatically activated to function. We measured their active protein form in tissue homogenate samples using ELISA kits that apply the Sandwich-ELISA principle (Elabscience Biotechnology, Rat MMP2- Cat#E-EL-R0618, and Rat MMP9- Cat#E-EL-R3021). The 96-well plates in these kits are pre-coated with antibodies specific for MMP-2 or MMP-9 (rat species). After adding standards or samples, a biotinylated detection antibody specific for rat MMP-2 or MMP-9 was added. The Avidin-Horseradish Peroxidase (HRP) complex was used for color development, where an Avidin-Horseradish Peroxidase

### Table 1 | Primer sequences for RT-qPCR used for the mRNA expression analysis in the present study.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primers</th>
<th>NCBI accession number</th>
<th>Amplicon size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zeb 1-Forward</td>
<td>GCCTGAACCTCAACCTAGTAA</td>
<td>NM_001308265.2</td>
<td>106</td>
</tr>
<tr>
<td>Zeb 1-Reverse</td>
<td>TCCGTGAACCTGGATGAAAAC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Zeb2- Forward</td>
<td>CTCTCTCATCTGCTCTACAG</td>
<td>NM_001033701.1</td>
<td>107</td>
</tr>
<tr>
<td>Zeb2- Reverse</td>
<td>GAGAAAAGACCCGTCTGATATT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vimentin- Forward</td>
<td>GCCTTGAAGCTGCTAATCA</td>
<td>NM_031140.1</td>
<td>111</td>
</tr>
<tr>
<td>Vimentin- Reverse</td>
<td>ATGGAGCAGGTCTGATATT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Catenin beta 1- Forward</td>
<td>TATGCCTGCTGGTCTATT</td>
<td>NM_053357.2</td>
<td>98</td>
</tr>
<tr>
<td>Catenin beta 1- Reverse</td>
<td>TCTGCCTGAGGGAGGAACT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>B-actin- Forward</td>
<td>GACGTGGACATCGTAAGACC</td>
<td>NM_031144.3</td>
<td>113</td>
</tr>
<tr>
<td>B-actin- Reverse</td>
<td>CTAGGAGCCAGGGCAGTATCT</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Abbreviations: mRNA, messenger RNA; RT-qPCR, quantitative real-time PCR.
(HRP) conjugate was added to each well, then after several washing steps, a substrate solution was added for color development. The addition of a stop buffer terminated the enzyme-substrate reaction. A spectrophotometer set at 450 nm was used to measure the optical density (OD), and the concentration of MMP-2 or MMP9 in the samples was estimated using a standard curve. Concentration values were represented as ng/mg protein.

2.8 Immunohistochemistry for vimentin expression

The expression of vimentin in the liver tissue sections from the different stages was evaluated through immunohistochemistry. Paraffin-embedded liver tissue sections were deparaffinized by xylene and rehydrated several times. The endogenous peroxidase activity was blocked with hydrogen peroxide. Heat-induced antigen retrieval was conducted, followed by blocking the nonspecific binding sites with a protein-blocking buffer (bovine serum Albumin). Liver tissue sections were incubated overnight at 4°C with the primary antibody Anti-Vimentin Rabbit Polyclonal antibody (GeneTex, Cat# GTX100619). After several washing steps, sections were treated with a relevant secondary antibody for 90 min. The protein conjugate was detected using a streptavidin-biotin-peroxidase complex. Finally, the sections were treated with Diaminobenzidine (DAB) for color development. Negative control using IgG1 was conducted in parallel as a quality control check. All stained liver sections were inspected using an electric light microscope Olympus BH2, and images were captured for analysis.

2.9 Statistical analysis

Results of the present study are presented as mean ± standard deviations. The ANOVA test with the post-hoc Bonferroni test is used to evaluate the significant differences between each induced stage and the normal control and each stage to the preceding one. Pearson’s correlation analysis was employed to assess the relationship between the expression of H19 or MALAT1 and the levels of the tumor progression genes. The Pearson’s correlation coefficient (r) reflects the strength and direction of the relationship. Differences were considered significant with the following p values: *p < .05, **p < .01, ***p < .001, ****p < .0001.

3 RESULTS

3.1 Histopathological evaluation of the multistage hepatocarcinogenesis model

The liver tissue collected from control rats revealed a normal histological structure of the parenchymal cells, normal lobulation, and regular arrangement of portal triads (Figure 2A). On the other hand, the DEN/NMOR-induced rats showed marked histological alterations in a stepwise pattern during the experiment. Liver sections of the stage I group (4 weeks of DEN-NMOR administration) revealed hepatocellular steatosis (Figure 2B), variable degrees of bile duct hyperplasia, fibrosis (pericholangiolar), cytoplasmic eosinophilia, some lymphoplasmacytic and few neutrophils infiltrate. For stage II liver sections (8 weeks of DEN-NMOR administration), cellular atypia, karyomegalic hepatocytes, and multinucleated hepatocytes were the prominent findings (Figure 2C). At 12 weeks of DEN-NMOR administration (representing stage III), animals showed, in addition to the above alterations, the development of altered foci of cellular atypia, including basophilic, clear, and amphiphilic cellular types. These foci are speculative preneoplastic lesions varied from hardly tangible to tinctorial and cyt morphological discrete lesions. They were classified based on hematoxylin and eosin tinctorial properties and cyt morphological features. The clear cell foci were composed of enlarged cells with featured clear cytoplasm, small dense nuclei, some eosinophilic cells, anisonucleosis, and anisokaryosis (Figure 2D). The basophilic foci usually comprise smaller cells that are arranged in tortuous cords. Moreover, enlarged cells were also noticed and often arranged in clumps or long bands with stripped patterns (Figure 2E).

Amphiphilic foci showed an obvious tinctorial variation of basophilic and eosinophilic cells with clear anisocytosis and anisokaryosis. At Week 16 of the DEN-NMOR administration (Stage IV), animals showed a severe chronic inflammatory reaction, marked bile duct proliferation with clustering and insinuation of the proliferated cells among the parenchymal cells, dilated lymphatics, and hepatocellular eosinophilia, hypertrophy with marked anisonucleosis and anisokaryosis (Figure 2F). Some animals showed cirrhotic activity with corrugated capsules and nodular hyperplasia (Figure 2G). The first incidence of hepatocellular neoplasia (hepatocellular adenoma) was noticed in this group. Adenomas appeared as well-delineated, unencapsulated nodules composed of hepatic cells that varied in size and tinctorial characteristics. These nodules lacked a normal lobular architecture with inapparent central veins and portal triads (Figure 2H). The neoplastic cells showed cellular atypia, and variation in tinctorial intensity, some of which showed cytoplasmic basophilia or vacuolated cytoplasm. An increase in the nucleus-to-cytoplasm ratio was detected and sometimes revealed focal attempts for trabeculae formation. Multinucleated hepatocytes were observed in some adenomas. The neoplastic cell nuclei were pleomorphic, showing coarsely clumped chromatin and large nucleoli.

Hepatic cell carcinomas (HCCs) were observed following 20 weeks of DEN-NMOR administration, representing stage V in our study. The neoplastic cells in those carcinomas approximated the appearance of normal hepatocytes but varied in size and tinctorial characteristics and grew either in trabecular or acinar patterns. In the trabecular pattern, the cells were arranged in thick cords (2–3 thick) (Figure 2I) and showed variation in tinctorial intensity, marked anisokaryosis, anisocytosis, and karyomegalic cells. Sometimes these trabeculae alternate with diluted sinusoids that form blood lakes in some cases (Figure 2J) with the same cellular pleomorphicism. While in the acinar pattern, the neoplastic hepatocytes are arranged in a variable acinar structure, forming a single layer around a central clear space (Figure 2K). Usually, those acini are
interspersed with areas that have trabecular architecture (Figure 2L). One case showed Mixed hepatocellular and cholangiocellular carcinoma (Figure 2M) characterized by malignant hepatocytes and cholangiolar epithelium. The hepatic cells formed islands and duct-like structures and appeared karyomegalic with marked anisokaryosis and anisonucleosis (Figure 2N). The biliary epithelium appeared markedly proliferated, forming ducts and insinuating among the hepatic cells with marked oval cell hyperplasia (Figure 2O).
3.2 Aberrant expression of H19 and MALAT1 during the stepwise hepatocarcinogenesis

The expression analysis of H19 and MALAT1 revealed that their levels changed in parallel with the induced stepwise hepatocarcinogenesis (Figure 3). A dynamic gradual elevation in their expression was detected by stage development over the time course of the experiment, reaching a marked significant elevation \( (p < .0001, p < .01) \) in the last two stages representing hepatocellular adenoma and carcinoma as compared to normal control. Regarding the expression analysis of H19, the relative expression value for each stage was calculated as follows: stage I = 0.98 ± 0.24, stage II = 1.16 ± 0.6, stage III = 2.79 ± 0.93, stage IV = 2.57 ± 0.9, and stage V = 3.41 ± 1.23. Meanwhile, for MALAT1, the relative expression value for each stage was calculated as follows: stage I = 2 ± 0.49, stage II was 2.44 ± 0.93, stage III was 2.48 ± 0.6, stage IV was 3.16 ± 0.69, and stage V = 3.55 ± 0.84.

All significance differences were related to the normal control group for H19 (0.91 ± 0.2) and MALAT1 (0.96 ± 0.3). Interestingly, we could not detect a significant difference in the expression of H19 or MALAT1 between each stage and the one preceding, except in H19 between stage III and stage II (Figure 3).

3.3 Expression pattern of ZEB1, ZEB2, β-catenin, vimentin, MMP2, and MMP9 in HCC stepwise development stages

According to the gene expression assessment, the increases in ZEB1 and ZEB2 were only significant at the late stages of hepatocarcinogenesis (Figure 4A,B). This result is reasonable given that ZEB1 and ZEB2 overexpression is commonly linked with poor prognosis. On the other hand, the elevation in the mRNA expression of vimentin and β-
FIGURE 4  Relative expression analysis of tumor progression biomarkers ZEB1, ZEB2, β-catenin, and vimentin and the concentration of MMP2, and MMP9 in the HCC-stepwise development stages. mRNA expression for the chosen tumor progression genes was evaluated by RT-PCT in the five induced stages. The dynamic elevation in their expression during stages was represented as the mean of relative expression ± SD from three independent experiments. The significant difference was evaluated for each stage compared with the control group and compared to the stage preceding it. Differences were considered significant at; *p < 0.05; **p < 0.01; ***p < 0.00; ****p < 0.0001.
Catenin was determined to be significant starting from the third stage, where preneoplastic lesions were detected, scoring a highly significant elevation \((p < .0001)\) in the last advanced stage where HCC was developed (Figure 4C,D).

Regarding the protein concentration of active MMP2 and MMP9, we detected an elevation in their levels starting from stage II, where hepatocytes started to transform and cellular atypia developed. The elevation of MMP2 and MMP9 continued dynamically in the later stages, reaching its highest at the final stage (Figure 4E,F).

### 3.4 Immunohistochemical detection of vimentin expression in the different stages of induction

Vimentin is critical for cell morphology, proliferation, and motility. Furthermore, a distinctive feature of establishing an EMT phenotype is the elevated expression of the mesenchymal markers vimentin. Therefore, in our work, we assessed the protein expression of vimentin in the stepwise stages of the hepatic carcinogenesis model (Figure 5). The immunohistochemistry staining revealed that vimentin expression increased in parallel with the progression of the carcinogenesis stages, beginning with cellular atypia (Figure 5B), then altered foci (Figure 5C,D), reaching a high expression in adenomas (Figure 5E,F), and trabecular and acinar carcinomas tissue sections (Figure 5G,H). A significant elevation of vimentin was detected in the final induction stage with the development of combined hepatocellular and cholangiocellular carcinomas (Figure 5I).

### 3.5 The correlation coefficient of H19 and MALAT1 with the tumor progression biomarkers

With the above-stated results, our next goal was to elucidate the correlation between the expression pattern of H19 and MALAT1 with the selected biomarkers involved in liver carcinogenesis. Pearson correlation analysis (Figure 6) revealed that the elevated expression pattern of H19 or MALAT1 were positively correlated with the stepwise increased levels of ZEB1, ZEB2, β-catenin, vimentin, MMP2, and MMP9. Although there was a significant positive correlation between all markers, the correlation between H19 or MALAT1 with ZEB1 was modest \((r = .438\) and \(.418\), respectively), and the correlation between H19 or MALAT1 with MMP2, MMP9, and vimentin was the highest. The relative expression of H19 scored a high positive correlation coefficient of \(r = .667\) with MMP2, \(r = .65\) with MMP9, and \(r = .677\) with vimentin. On the same line, MALAT1 scored a high positive correlation coefficient of \(r = .717\) with MMP2, \(r = .8\) with MMP9, and \(r = .69\) with vimentin (Figure 6).
Hepatocarcinogenesis is a complex process mediated by the accumulation of genetic and epigenetic alterations. Most studies on hepatocyte transformation and tumor development focused on the impact of protein-coding genes. However, growing evidence suggests that noncoding RNAs (including lncRNAs) play a central role in hepatocarcinogenesis. lncRNAs are involved in every step of gene expression regulation, including chromatin remodeling, transcriptional control, splicing regulation, mRNA stability, miRNA processing, and protein stability.

Pathogenesis of various diseases, including HCC, has been related to aberrant lncRNA biogenesis. During HCC transformation, the expression of particular lncRNAs is dysregulated. Considering previous research denoting that abnormal H19 expression is connected with inflammation, and given that HCC is most commonly caused by chronic inflammation, we sought to assess the expression pattern of H19 in the different stages of HCC development. Moreover, we assessed the expression pattern of another essential lncRNA linked to the hepatocarcinogenesis process, MALAT1.

In our study, we used a chemically induced carcinogenesis model that closely mimics the development of human HCC, with stepwise stages resembling fibrosis, cirrhosis, adenoma, and, eventually, hepatocellular carcinoma. Through this model, we addressed the genetic and epigenetic alterations involved in the stepwise carcinogenesis process. The expression of H19 and MALAT1 was dynamically upregulated by stage progression, indicating their involvement in liver carcinogenesis. The functional relevance of H19 in the carcinogenesis process is still debated; some studies support their carcinogenic activity, while others claim a tumor-suppressive impact. However, our study supports their oncogenic involvement in hepatocarcinogenesis. In line with our findings, the study by Gamaev et al. reported that lncRNA H19 was identified as pro-oncogenic during the progress of chronic inflammation-mediated HCC in a mouse model. According to this study, H19 enhances liver damage and lowers hepatocyte polyploidy. Regarding the studies that reported a tumor suppressive effect of H19, it is important to highlight that these studies investigated the levels of H19 in tumor tissues compared to their respective normal adjacent tissue or studied the function of H19 in cell lines that have been reported before to be contaminated with Hela cells. However, none of these studies investigated the expression pattern of H19 in the...
progression mode in which chronic inflammation preceded the development of HCC, like the present study.

One of the most studied IncRNA in cancer research is MALAT1, which is implicated in various liver disorders. To confirm the oncogenic involvement of MALAT1 in liver tumor induction, Peng et al. found that knocking down MALAT1 reduced HCC cell growth and viability via accelerating apoptosis and autophagy. Studies investigating the function of MALAT1 in HCC suggested its oncogenic function through upregulating the oncogenic SR protein splicing factor (SRSF1) and activating the mTOR pathway, or through acting as a molecular sponge for several tumor suppressor miRNAs like miR-146a, miR-613, miR-6887-3p, or miR-125a-3p leading to activation of the survival signaling pathways regulated by these miRNAs like PI3K/Akt/mTOR, Wnt/β-catenin, JAK2/STAT3, and FOXM1. Although numerous target genes and pathways for H19 and MALAT1 have been reported, the epithelial-mesenchymal transition (EMT) is the most affected pathway controlled by these two IncRNAs.

A growing body of evidence demonstrates that hepatic EMT is essential in the progression of malignant hepatocytes. The transcriptional factors ZEB1, ZEB2, and mesenchymal marker vimentin are the essential players identified to potentiate EMT and are involved in the carcinogenesis and metastasis of HCC. According to our data, ZEB1 and ZEB2 expressions were significantly elevated by the late induction stage. This finding stands to reason since their expression is frequently associated with the late transformation and invasiveness of the tumor. On the other hand, we detected a stepwise elevation in the mesenchymal marker vimentin by the gradual transformation of hepatocytes from preneoplastic lesions to adenoma and carcinoma.

Early epithelial to mesenchymal transition, tumor cells produce epithelial (cytokeratins) and mesenchymal (vimentin) markers. However, the mesenchymal markers become more prominent during tumor progression, while epithelial markers are repressed. It should be noted that vimentin is not regarded as a typical tumor progression marker since mature epithelial cells could also produce vimentin in response to stress.

The Wnt/β-catenin signaling pathway is another generic driver of HCC progression. Overexpression of β-catenin promotes the characteristics of cancer stem cells in HCC and raises their progression rate. Moreover, β-catenin inhibits apoptosis and promotes migration by inducing EMT and increasing levels of MMPs. Activation of β-catenin has been linked to both early and late stages of HCC. This was evident in our data, where the relative expression of β-catenin was significantly elevated in the stage representing preneoplastic lesions (Stage III) and increased in the successive stages representing adenoma and carcinomas.

Liver fibrosis is characterized by excessive extracellular matrix (ECM) deposition and enhanced matrix metalloproteinase activity (ECM degrading enzymes). Activation of MMPs is observed in the vast majority of carcinomas, especially HCC, and frequently associated with poor prognosis. MMPs are structurally classified into numerous subtypes, among which MMP 2 and 9 are linked to tumor growth, clinical stages, and poor prognosis. MMP2 and MMP9 activity were observed in our study at the early stages of induction and significantly increased with stage development, reaching almost a two-fold rise with HCC induction compared to the early stage.

IncRNAs could contribute in regulating the above-mentioned EMT-related genes through epigenetic modification, regulation of gene transcription, or post-translational modification. H19 could influence the expression of many EMT-related genes by functioning as a competitive endogenous RNA or mRNA decoy, thus abolishing the endogenous suppressive effect of miRNAs that target EMT marker genes. For example, in colorectal cancer, H19 acted as a competing endogenous RNA for miR-138 and miR-200a, antagonizing their activity and enhancing the expression of their endogenous targets Vimentin, ZEB1, and ZEB2, all of which are EMT signature genes. In gastric cancer, H19 was reported to compete with miR-141 and thus enhance ZEB1 expression. H19 and miR-141 knockdown lowered ZEB1 expression, whereas H19 overexpression increased ZEB1. Suggesting that H19 can control the miR-141 target gene ZEB1. MALAT1 was shown to have the same functional impact. It is linked to HCC cellular proliferation, survival, migration, and invasion by sponging the activity of many miRNAs, including miR-200a, a putative tumor suppressor in EMT, and miR-143-3p that targets ZEB1. Previous loss of function studies suggested that targeting MALAT1 could be a potential anticancer approach. MALAT1 silencing decreased the expression levels of β-catenin, vimentin, Twist1, Stat3, c-myc, and CK1, hence inhibiting the oncogenicity of HCC. Based on these prior findings, we infer that IncRNAs H19 and MALAT1 are associated with activating tumor progression genes that promote hepatocarcinogenesis. This association was confirmed in our study by the positive correlation coefficient between H19 or MALAT1 and the tumor progression markers ZEB1, ZEB2, β-catenin, vimentin, MMP2, and MMP9.

All results presented in our study should be interpreted carefully and confirmed by clinical studies on large and independent series of patients with various stages of liver cancer.

5 | CONCLUSION

In our study, we used a chemically induced hepatocarcinogenesis model that reproduces the sequential stages of initiation, promotion, malignant conversion, and progression. Using this model, we demonstrated that IncRNAs H19 and MALAT1 are implicated in each stage of the pathogenesis of HCC, with an elevated dynamic expression pattern as the carcinogenesis stages proceed. Furthermore, we detected a strong correlation between H19 or MALAT1 expression and levels of MMP2, MMP9, and vimentin expression, suggesting that epigenetic regulation during HCC stepwise development is connected with alterations in the tumor progression genes.

AUTHOR CONTRIBUTIONS

Sherien M. El-Daly developed the idea and planned the work. Sherien M. El-Daly, Mona A. El-Bana, Dalia Medhat, Sahar S. Abd El-
Rahman, and Yasmin Abdel Latif performed the required experiments. Sherien M. El-Daly, Sahar S. Abd El-Rahman, Mona A. El-Bana, Dalia Medhat, and Yasmin Abdel Latif participated in data acquisition and interpretation. Sherien M. El-Daly wrote the manuscript in consultation with the rest of the authors.

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CONFLICT OF INTEREST STATEMENT
The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT
All data generated during this study are available from the corresponding author upon reasonable request and with permission of the National Research Centre.

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