SEC62 and SEC63 Expression in Hepatocellular Carcinoma and Tumor-Surrounding Liver Tissue

Markus Casper^a Maximilian Linxweiler^b Johannes Linxweiler^c
Richard Zimmermann^b Matthias Glanemann^c Frank Lammert^a
Susanne N. Weber^a

^aDepartment of Medicine II, Saarland University Medical Center, Homburg, Germany; ^bDepartment of Medical Biochemistry and Molecular Biology, Saarland University Medical Center, Homburg, Germany; ^cDepartment of Urology and Pediatric Urology, Saarland University Medical Center, Homburg, Germany

Keywords

Chronic liver disease · Endoplasmic reticulum · Gene expression · Hepatocarcinogenesis · Immunohistochemistry · Intracellular signaling · Liver cancer · Liver tumor

Abstract

Introduction: The endoplasmic reticulum transmembrane proteins Sec61, Sec62, and Sec63 are responsible for the intracellular trafficking of precursor proteins and affect intracellular signaling. SEC62 overexpression has been linked to various human cancers. Our aim was to investigate SEC62 and SEC63 expression in hepatocellular carcinoma (HCC) and surrounding liver tissue. **Patients and Methods:** Primary liver tissue was collected from 11 consecutive patients (70 ± 9 years; 10 men) who underwent HCC resection. In the HCC and the tumor-surrounding liver tissue we investigated SEC62 und SEC63 mRNA expression using quantitative real-time PCR. For Sec62, immunohistochemistry was performed. **Results:** SEC62 and SEC63 total mRNA contents were significantly ($p = 0.001$) higher in HCCs (C_T 22.5 ± 0.4 and 22.6 ± 0.3) when compared to the surrounding tissue (C_T 24.6 ± 0.6 and 25.1 ± 0.9). Using the comparative C_T method, SEC62 and SEC63 expression in HCC was increased 5- and 8.1-fold, respectively, in comparison to surrounding tissue. For Sec62 immunohistochemistry, the mean immunoreactive scores

(IRS) were 7.9 ± 2.9 for HCC and 4.8 ± 1.2 for non-tumorous liver ($p = 0.027$). The mean IRS in HCC were 5.7 ± 3.5 and 8.9 ± 2.3 for patients without ($n = 3$) and with tumor recurrence ($n = 8$), respectively. **Conclusions:** Overexpression of SEC62 and SEC63 is a common feature of HCC. The role of Sec62 as a prognostic marker for tumor recurrence after surgery and its potential role in treatment stratification must be addressed in future studies.

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Introduction

Hepatocellular carcinoma (HCC) is the sixth most common cancer worldwide with increasing incidence rates, and one of the leading causes of cancer-related death [1, 2]. Cirrhosis is the strongest risk factor for HCC development and often limits treatment options because of significantly impaired liver function. However, 20% of HCCs develop into non-cirrhotic livers, especially in patients with chronic hepatitis B virus infection or non-alcoholic fatty liver disease [3].

Over the last decades our understanding of hepatocarcinogenesis has rapidly improved. The molecular and genetic landscape of HCC is very heterogeneous and multiple signaling pathways and genes as well as microRNAs

have been found to be involved [4]. The trafficking of precursor polypeptides into or across the endoplasmic reticulum (ER) membrane is essential for the biogenesis of transmembrane and secretory proteins [5]. The ER transmembrane proteins Sec61 complex, Sec62, and Sec63 are core components of the protein translocation machinery responsible for the co- and posttranslational protein transport [5, 6]. Moreover, Sec proteins can affect intracellular signaling: Sec62 induces the process of recovery of cells from ER stress, and Sec61 complex also acts as a calcium leak channel [6, 7].

Overproduction of Sec proteins and mutations in the corresponding genes have been linked to human disease: mutations in *SEC63* are associated with autosomal-dominant polycystic liver disease [8], and *SEC63* may act as a tumor suppressor gene. *SEC63* frame-shift mutations are common in microsatellite unstable colonic and gastric cancers and small-bowel carcinomas [6, 9, 10]. The strongest evidence for a causative role in carcinogenesis exists for Sec62. *SEC62* overexpression in tumor tissue (mRNA and/or protein level) was demonstrated for prostate cancer [11, 12], lung cancer, thyroid cancer [13], head and neck squamous cell carcinoma [14], cervical cancer [15], and breast cancer [16].

High *SEC62* expression in HCC tissue and peripheral blood mononuclear cells of patients with HCC has been shown to indicate increased risk for early HCC recurrence after resection [17, 18]. So far, no study has analyzed *SEC62* and *SEC63* expression levels in HCC and tumor adjacent liver tissue.

Materials and Methods

Patients

Eleven consecutive patients with HCC who underwent resection of HCC at the Saarland University Medical Center were prospectively recruited for the study. The follow-up time for recurrence was 5 years for each patient. All of them had non-metastatic disease BCLC (Barcelona clinic liver cancer) stage A. Only 3 patients had histologically confirmed liver cirrhosis (Child-Pugh class A). Table 1 provides the patient characteristics. Immediately after resection representative HCC tissue samples were stored in RNAlater in the operating room (Qiagen, Hilden, Germany). Corresponding tissue samples were also collected from tumor-surrounding non-tumorous liver tissue. Afterwards all samples were stored at -80°C . The surgical specimen was stored in formalin and sent for histopathological assessment.

Gene Expression Analyses

RNA was isolated from frozen livers using an RNeasy Mini Kit (Qiagen). One microgram of RNA was transcribed to cDNA using the High-Capacity cDNA Reverse Transcription kit (Life Technologies, Darmstadt, Germany). Expression of *SEC62* (Hs00162786_m1) and *SEC63* (Hs00273093_m1) was determined on the Taqman[®] 7500 Fast Real-Time PCR system (Life Technologies). The following genes were used as endogenous controls: *GAPDH* (Hs02758991_g1), *18S* (Hs99999901_s1), and *HMBS*

Table 1. Baseline characteristics of the study cohort

Age, years	70±9
Male/female	10/1
Liver disease	
NAFLD	4
Alcoholic liver disease	3
Hepatitis B	2
Hepatitis C	2
Liver fibrosis stage	
F1–3	8
F4	3
Liver function tests	
ALT, U/L	59.6±48.7
Bilirubin, U/L	0.9±0.3
Albumin, g/L	42.9±3.0
INR	1.1±0.1
Tumor characteristics	
Multifocal/unifocal disease	3/8
Diameter of largest nodule, mm	51±27
Grading G1/G2/G3	1/9/1
Tumor recurrence	7

Data are presented as the mean ± SD or *n*. ALT, alanine aminotransferase; INR, international normalized ratio; NAFLD, non-alcoholic fatty liver disease.

(Hs00900675_m1) and *SRSF4* (Hs00609396_g1; Life Technologies). Relative expressions of *SEC62* and *SEC63* were calculated using the $\Delta\Delta\text{C}_\text{T}$ method [19] and normalized to controls (surrounding liver tissue).

Immunohistochemistry

Tissue samples (HCC and tumor-free adjacent tissue) from each of the 11 patients were previously hematoxylin and eosin stained and evaluated by a pathologist. Sections were transferred onto Superfrost Ultra Plus Microscope Slides (Menzel-Gläser, Braunschweig, Germany) and incubated at 37°C overnight. On deparaffinization, heat-induced epitope retrieval was performed by microwave treatment in 10 mmol of citrate buffer (pH 6.0), and nonspecific protein binding sites were blocked by incubation in blocking solution (80 mL of 0.1 M Tris-HCl, pH 7.2, 3 g of bovine serum albumin, and 20 mL of FBS; Sigma-Aldrich Chemie GmbH) for 30 min at room temperature. Subsequently, primary antibody incubation was performed with a 1:200 solution (diluted in PBS/0.3% BSA) of an affinity purified Sec62 polyclonal rabbit antibody (provided by the Institute of Medical Biochemistry and Molecular Biology) for 1 h at room temperature. The antibody was directed against the carboxy terminal undecapeptide of human Sec62 (plus an additional amino terminal cysteine) and previously demonstrated to be specific under denaturing and native conditions [13]. Each staining series included a positive control and two negative controls (either without primary antibody or with incubation in a 1:200 solution of the pre-immune serum). Visualization was performed using the DAKO Real Detection System (DAKO, Glostrup, Denmark) according to the manufacturer's instructions, and slides were counterstained with hematoxylin. Sec62 protein levels were evaluated using the immunoreactive score (IRS) according to Remmele and Stegner [20] as a well-established and unbiased semiquantitative validation system. In this system, staining intensity is classified as no staining (0), weak (1), intermediate (2), or strong (3). The number of stained cells is clas-

Fig. 1. Sec62 protein levels in HCC and adjacent liver tissue. **A** Representative images (original magnification, $\times 100$) of Sec62 immunostained tissue from 3 patients (HCC on the left and corresponding non-tumorous liver on the right). A higher Sec62 protein level in HCCs is indicated by stronger red staining. In patients P1–P3 the IRS was 12, 9, and 9 for the HCC and 6 for the surrounding liver tissue. **B** Sec62 protein levels of HCC and surrounding liver tissue in individual patients quantified with the IRS according to Remmele and Stegner.

sified as no stained cells (0), below 10% (1), 10–50% (2), 51–80% (3), or more than 80% (4). The product of the scores for staining intensity and number of stained cells is defined as the IRS. Scores of 0–2 were valued as negative, 3–4 as weak, 6–8 as moderate, and 9–12 as strong.

Statistical Analysis

For statistical analysis, Wilcoxon matched-pairs signed-rank tests were used with Prism 5.0 software (GraphPad, San Diego, CA, USA). $p < 0.05$ was considered statistically significant.

Results

Sec62 Immunohistochemistry in HCC and Adjacent Liver Tissue

Sec62 protein levels were evaluated by Sec62 immunohistochemistry and quantified using the IRS by Remmele and Stegner. There was an elevated Sec62 protein level in HCCs as compared to the adjacent non-tumorous tissue in 9 out of 11 patients (Fig. 1). Sec62 protein levels were lower in HCC or equal in HCC and non-tumorous tissue

Fig. 2. SEC62 and SEC63 mRNA levels in HCC and surrounding liver tissue. Raw C_T values for SEC62 (A1) and SEC63 (B1). Lower C_T values indicate higher mRNA levels. The relative expression in comparison to the surrounding tissue using the comparative C_T method is shown for SEC62 (A2) and SEC63 (B2). HCC is indicated by black bars; grey bars represent the surrounding tissue.

in one patient each. The mean IRS was 7.9 ± 2.9 for HCC and 4.8 ± 1.2 for non-tumorous liver. The Wilcoxon matched-pairs signed-rank test revealed a significant ($p = 0.027$) difference in Sec62 protein levels between HCC and non-tumorous tissue.

In 8 out of 11 patients, tumor recurrence occurred after resection. The mean time interval between initial treatment and HCC recurrence was 31 months (range 7–64). The three patients without tumor recurrence included the 2 patients who showed lower or identical Sec62 protein levels in their HCC when compared to non-tumorous liver tissue (Fig. 1; patients 5, 7, 9). The mean IRS in HCC was 5.7 ± 3.5 and 8.9 ± 2.3 for patients without and with recurrence, respectively. Seven of 8 patients (87.5%) with an IRS ≥ 8 developed HCC recurrence.

SEC62 and SEC63 mRNA Levels in HCC and Adjacent Liver Tissue

Using quantitative real-time PCR SEC62 and SEC63 mRNA levels were measured in the 11 HCCs and tumor-free surrounding liver tissue. SEC62 total mRNA contents were significantly ($p = 0.001$) higher in HCC (C_T

22.5 ± 0.4) as compared to the surrounding tissue (C_T 24.6 ± 0.6). For SEC63, we also found a significant ($p = 0.001$) upregulation of mRNA levels in HCC (C_T 22.6 ± 0.3) in comparison to surrounding liver tissue (C_T 25.1 ± 0.9). In order to calculate the relative expressions of SEC62 and SEC63 in HCC compared to matched surrounding tissue, we converted C_T values to the linear form using the comparative C_T method (n -fold difference = $2^{-(\text{mean } C_T \text{ HCC} - \text{mean } C_T \text{ surrounding liver tissue})}$). For HCC, a 5-fold increase (± 3.5) of SEC62 expression and an 8.1-fold (± 5.1) increase of SEC63 expression was observed. Figure 2 plots the values for individual patients.

mRNA Levels of Housekeeping Genes in HCC and Surrounding Liver Tissue

For the housekeeping genes GAPDH (glyceraldehyde-3-phosphate dehydrogenase), 18s (18s ribosomal RNA), HBMS (hydroxymethyl-bilane synthase), and SFRS4 (arginine/serine-rich splicing factor 4) mRNA levels were measured in HCC and tumor-surrounding liver tissue. As shown in online supplementary Figure 1 (see www.karger.com/doi/10.1159/000513293 for all on-

line suppl. material), except for *SFRS4* and *HMBS* in HCC patient 8, mRNA levels of the tested housekeeping genes were elevated in HCCs. The mean C_T values (HCC vs. surrounding liver tissue) were 20.8 ± 0.5 and 22.8 ± 1.1 for *GAPDH*, 10.7 ± 0.9 and 14.7 ± 4.9 for *18s*, 27.9 ± 0.7 and 30.4 ± 1.8 for *HMBS*, and 26.6 ± 0.7 and 28.0 ± 1.9 for *SFRS4*. Thus, mRNA levels were significantly higher in HCC for all four evaluated housekeeping genes (*GAPDH*, $p = 0.002$; *HMBS*, $p = 0.002$; *SFRS4*, $p = 0.0029$; *18s*, $p = 0.001$).

Relative Quantification of *SEC62* and *SEC63* Using Different Housekeeping Genes

SEC62 and *SEC63* mRNA contents were normalized to the four tested housekeeping genes as reference genes, and an n -fold increase in tumor relative to tumor-free tissue ($2^{-\Delta\Delta C_T}$) was calculated (online suppl. Fig. 2). This resulted in inconsistent results for relative quantification of *SEC62* and *SEC63* mRNA, due to variant expression of the four housekeeping genes in HCC and tumor-free tissue, which were not in line with the results of Sec62 immunohistochemistry.

Discussion

SEC62 overexpression in tumor tissue in comparison to non-tumorous tissue has been described in extrahepatic cancers [11–16]. The *SEC62* gene with potential oncogenic functions is located on chromosome 3q26.2. Interestingly, gains on chromosome arm 3q were also observed in HCC [21, 22]. Here, for the first time, we investigated Sec62 protein and *SEC62* mRNA levels in HCC and surrounding liver tissue in Caucasians. Immunohistochemistry on paraffin-embedded tumor specimens showed upregulation of the Sec62 protein. This is in line with data previously reported for other cancers [11–16] and supported by the corresponding changes of *SEC62* mRNA expression.

Whereas mutations in *SEC63* were described in microsatellite unstable carcinomas, gene expression has not been studied so far. Here, we have shown that *SEC63* mRNA is overexpressed in HCC. However, this should be evaluated in larger series, and the functional roles of Sec63 in carcinogenesis have yet to be defined.

For gene expression experiments, normalization is used to account for different amounts of starting mRNA or mRNA quality, variable enzymatic efficiencies during cDNA synthesis or differences between tissues or cells in overall transcriptional activities. One common approach is the amplification of a housekeeping gene and the normalization of expression for the target gene using the $\Delta\Delta C_T$ method [19]. However, it has been shown that genes typically used as reference genes are differentially

expressed in tumorous and non-tumorous tissue [23] and can thus confound data as well. Here, in addition to the common housekeeping genes *GAPDH* and *18s*, we evaluated the genes *SFRS4* and *HMBS* that have been previously reported to show stable expression levels in HCC and surrounding liver tissue [24, 25]. In contrast to these reports, in our series *SFRS4* and *HMBS* as well as *GAPDH* and *18s* were upregulated in HCCs. Since the quantity of the starting material may be the most relevant confounding factor, and we used stable RNA concentrations after measurement, comparison of raw C_T values may be appropriate in this setting.

It has been shown that high expression of Sec62 in HCC tissue and peripheral blood mononuclear cells is an independent risk factor for HCC recurrence after liver resection [17, 18]. In line with these observations, Sec62 was shown to be a prognostic biomarker in prostate cancer, head and neck-cancer, and lung cancer, as well as breast cancer [12, 13, 15, 16, 26]. Several studies indicate a role of Sec62 in cell migration and invasion processes [13, 14, 18], which may explain its role as a prognostic factor. In our study, we also found stronger *SEC62* expression confirmed by immunohistochemistry in patients with recurrent HCC. Interestingly, in the only 2 patients without *SEC62* overexpression in HCC in comparison to surrounding tissue, HCC did not recur. An IRS ≥ 8 was observed in 33% of patients without but 88% of patients with recurrence. However, due to the small study sample, these results and the cut-off value should be studied in larger cohorts.

Besides its potential role as a prognostic marker, Sec62 may also be a therapeutic target. One potential mechanism is the treatment with calmodulin antagonists (e.g., trifluoperazine; TFP) antagonizing the calmodulin-mediated Sec61 closure and thus increasing the passive Ca^{2+} efflux from the ER lumen, compatible with a functional Sec62 knockdown [6, 26, 27]. Interestingly, there is evidence for a potential therapeutic effect of TFP in hepatoma cell lines [28, 29]. The most recent study showed that treatment of HCC cell lines with TFP impaired tumor cell vitality, invasion, and migration, and induced apoptosis [29]. TFP might thus be an interesting antitumor agent in *SEC62* overexpressing HCC. In particular, its potential role in avoiding tumor recurrence after curative resection could be investigated in the adjuvant setting.

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Statement of Ethics

The study was approved by the local ethics board (Ethikkommission der Ärztekammer des Saarlandes; approval No. 79/12). The patients provided written informed consent, and the study was conducted in accordance with the Declaration of Helsinki.

Conflict of Interest Statement

The authors declare that they have no conflicts of interest or financial interests related to the manuscript.

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Author Contributions

All authors fulfill the criteria of authorship and approved the final version of the manuscript. M.C.: study design, interpretation of the results, manuscript preparation. M.L.: immunohistochemistry, interpretation of the results, revision of the manuscript. J.L.: immunohistochemistry, interpretation of the results, revision of the manuscript. R.Z.: interpretation of the results, revision of the manuscript. M.G.: patient and tissue recruitment, revision of the manuscript. F.L.: study design, interpretation of the results, revision and editing of the manuscript. S.N.W.: mRNA expression analysis, data interpretation, manuscript preparation.

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