Original Article

Diagnostic and Prognostic Profiling of Nucleocytoplasmic Shuttling Genes in Hepatocellular Carcinoma

(nucleocytoplasmic transport / karyopherin / molecular profiling / cancer / hepatocellular carcinoma)

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Abstract. One of the key features of eukaryotic cells is the separation of nuclear and cytoplasmic compartments by a double-layer nuclear envelope. This separation is crucial for timely regulation of gene expression, mRNA biogenesis, cell cycle, and differentiation. Since transcription takes place in the nucleus and the major part of translation in the cytoplasm, proper distribution of biomolecules between these two compartments is ensured by nucleocytoplasmic shuttling proteins – karyopherins. Karyopherins transport biomolecules through nuclear pores bidirectionally in collaboration with Ran GTPases and utilize GTP as the source of energy. Different karyopherins transport different cargo molecules that play important roles in the regulation of cell physiology. In cancer cells, this nucleocytoplasmic transport is significantly dysregulated to support increased demands for the import of cell cycle-promoting biomolecules and export of cell cycle inhibitors and mRNAs. Here, we analysed genomic, transcriptomic and proteomic data from published datasets to comprehensively profile karyopherin genes in hepatocellular carcinoma. We have found out that expression of multiple karyopherin genes is increased in hepatocellular carcinoma in comparison to the normal liver, with importin subunit α-1, exportin 2, importin subunit β-1 and importin 9 being the most over-expressed. Moreover, we have found that increased expression of these genes is associated with higher neoplasm grade as well as significantly worse overall survival of liver cancer patients. Taken together, our bioinformatic data-mining analysis provides a comprehensive genomic and transcriptomic landscape of karyopherins in hepatocellular carcinoma and identifies potential members that could be targeted in order to develop new treatment regimens.

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Abbreviations: ARID1A – AT-rich interactive domain-containing protein 1A, ARID2 – AT-rich interactive domain 2, AXIN1 – Axin 1, CAN – copy number alteration, CREB – cAMP response element-binding protein, CSE1L – chromosomal segregation 1 like, CTNNB1 – catenin β 1, ERK2 – extracellular signal-regulated kinase 2, FDR – false discovery rate, FGFR4 – fibroblast growth factor receptor 4, HCC – hepatocellular carcinoma, INM – inner nuclear membrane, IPO5 – importin 5, IPO7 – importin 7, IPO9 – importin 9, KNPA6 – karyopherin subunit α6, KPNA – karyopherin α, KPNA2 – karyopherin subunit α2, KPNB – karyopherin β, KPNB1 – karyopherin subunit β2, NBS1 – nijmegen breakage syndrome protein 1, NES – nuclear export signal, NLS – nuclear localization signal, NPCs – nuclear pore complexes, ONM – outer nuclear membrane, RPL5 – ribosomal protein L5, RPS6KA3 – ribosomal protein S6 kinase α3, RPS7 – ribosomal protein S7, Ran – Ras-related GTPase nuclear protein, RanGEF – Ran guanine nucleotide-exchange factor, S6Kα3 – ribosomal protein S6 kinase 2, SMAD3 – mothers against decapentaplegic homolog 3, SREPB2 – sterol regulatory element-binding protein 2, TCGA – The Cancer Genome Atlas, TERT – telomerase reverse transcriptase, XPO1 – exportin 1, XPO7 – exportin 7.

Introduction

One of the defining characteristics of eukaryotic cells is the segregation of nuclear and cytoplasmic compartments (Dingwall and Laskey, 1992). This separation between the cytoplasm and nucleus is ensured by a semipermeable nuclear envelope that allows selective movement of materials between these two compartments (Stewart, 2022). The nuclear envelope, which is formed by closely opposed lipid bilayers, comprises an outer (ONM) and an inner (INM) nuclear membrane. The ONM is oriented towards the cytoplasm and is a direct extension of the endoplasmic reticulum, while the INM encloses the nucleoplasm (De Magistris and Antonin, 2018). The nuclear membrane secures the regulation of the import and export of nucleic acids and proteins from the nucleus to the cytoplasm and vice versa (Macara, 2001). The process of nuclear import and export plays a crucial role in determining the subcellular localization of various

macromolecules, including transcription factors, histones, and cell cycle regulators (Lu et al., 2021). These macromolecules are essential for numerous cellular processes, such as gene expression regulation (Stewart, 2022), cell cycle (Macara, 2001), DNA repair and maintenance (Kirby et al., 2015), signal transduction (He et al., 2010), or pre-ribosomal subunit transportation (Junod et al., 2023).

Molecular trafficking across the nuclear envelope is facilitated by the highly selective nuclear pore complexes (NPCs), which play a key role in ensuring the accurate localization of macromolecules in both directions (Çağatay and Chook, 2018). NPCs have an annular scaffold general structure with 8-fold rotational symmetry consisting of three rings: cytoplasmic, nucleocytoplasmic, and inner ring, which together form a central aqueous channel. NPCs are composed of approximately 30 different types of nucleoporins and many of them contain long intrinsically disordered domains facing into the central channel that possess typical sequence repeats rich in phenylalanine (F) and glycine (G) residues separated by short linkers and are known as FG-nucleoporins. These domains create a dense meshwork preventing free diffusion of macromolecules between the nuclear and cytoplasmic compartments (Rout et al., 2000; Frey et al., 2006). The permeability barrier formed by FG-nucleoporins in the NPCs is a crucial selective control for nucleocytoplasmic transport (Li et al., 2016). Additionally, nuclear pores associate with filamentous protein structures that extend from both the cytoplasmic and nucleoplasmic sides, which are organized into a structure called a nuclear basket on the nucleoplasmic side (Matsuura and Stewart, 2005; Christie et al., 2016; Li et al., 2021).

The transport of molecules smaller than 40 kDa across the nuclear envelope is enabled by passive diffusion. However, the high-molecular weight macromolecule transport is mediated by active transport. This is facilitated by specialized transport proteins called karyopherins (Stelma et al., 2016; Timney et al., 2016; Çağatay and Chook, 2018). Karyopherins are classified into two functional families: karyopherins α (KPNA) and karyopherins β (KPNB) (Table 1). Each member of these families has the ability to interact with specific cargo proteins or RNAs (Stelma et al., 2016). The karyopherin β family mediates the majority of transport of macromolecules across the NPCs into the nucleus (β-importins) and out of the nucleus (exportins) (Chook and Süel, 2011; Kimura and Imamoto, 2014; Soniat and Chook, 2015; Wing et al., 2022). β-Importins and exportins recognize specific signals, known as the nuclear localization signal (NLS) or the nuclear export signal (NES), within the cargo proteins (Çağatay and Chook, 2018). On the other hand, members of the karyopherin α family serve as adaptors binding the nuclear localization signal present in cargo proteins as well as members of the karyopherin β family (Moroianu et al., 1995; Conti et al., 1998). Import of the macromolecules into the nucleus is ensured either by direct interaction with β-importins or with the help of α-importins (Imamoto et al., 1995; Görlich and Mattaj, 1996). α-Importins are attached to the cargo by binding the classical NLS of the cargo and further create a complex with β-importins, which is translocated through the NPCs by binding the FG-nucleoporins (Radu et al., 1995; Rexach and Blobel, 1995; Wing et al., 2022). However, some cargoes contain a non-classical NLS, which is recognized and bound directly by β-importins without the adapter α-importins (Kim et al., 2017). The family of small Ras-related GTPase (Ran) regulates karyopherin-mediated nuclear transport and the assembly and disassembly of karyopherin-cargo complexes. During the import phase, the importin-cargo complex is formed and subsequently translocated through the NPC. In the nucleoplasm, GTP-bound Ran (RanGTP) binds to the importin-cargo complex, leading to its dissociation. Consequently, the RanGTP-bound importin is exported to the cytoplasm. Notably, the recycling of importin-α subunits is facili-

Table 1. List of karyopherin genes in the human genome

Gene name	Uniprot Protein name	Function
Karyopherin α family		
KPNA1	importin subunit α-5	Nuclear import adaptors
KPNA2	importin subunit α-1	Nuclear import adaptors
KPNA3	importin subunit α-4	Nuclear import adaptors
KPNA4	importin subunit α-3	Nuclear import adaptors
KPNA5	importin subunit α-6	Nuclear import adaptors
KPNA6	importin subunit α-7	Nuclear import adaptors
KPNA7	importin subunit α-8	Nuclear import adaptors
Karyopherin β family		
KPNB1	importin subunit β-1	Nuclear import
IPO4	importin 4	Nuclear import
IPO5	importin 5	Nuclear import
IPO7	importin 7	Nuclear import
IPO8	importin 8	Nuclear import
IPO9	importin 9	Nuclear import
IPO11	importin 11	Nuclear import
IPO13	importin 13	Bidirectional transport
TNPO1	transportin 1	Nuclear import
TNPO2	transportin 2	Nuclear import
TNPO3	transportin 3	Nuclear import
XPO1	exportin 1	Nuclear export
NXF1	nuclear RNA export factor 1	Nuclear export
XPOT	exportin T	Nuclear export
XPO4	exportin 4	Bidirectional transport
XPO5	exportin 5	Nuclear export
XPO6	exportin 6	Nuclear export
XPO7	exportin 7	Nuclear export
CSE1L	exportin 2	Nuclear import (exporting KPNα subunits)

tated by exportin 2, while the export of importin-β subunits does not require exportin 2 (Kim et al., 2017). In the process of export, exportins recognize NES in the cargo and subsequently form a complex with RanGTP in the nucleoplasm. This exportin-cargo-RanGTP complex then binds to NPCs and passes through the channel. Once in the cytoplasm, RanGTP is hydrolysed by RanGAP, leading to the dissociation of the complex. The resulting GDP-bound Ran (RanGDP) is then reimported back into the nucleoplasm with the assistance of nuclear transport factor 2 and loaded with GTP via Ran guanine nucleotide-exchange factor RanGEF (Kau et al., 2004; Stewart, 2007; Wente and Rout, 2010) (Fig. 1).

The proper localization of nuclear-cytoplasmic cargoes is essential for cells to execute their normal functions. A number of these cargoes are crucial regulators of the cell cycle, and dysregulation of their nuclear-cytoplasmic transport has been shown to support oncogenic transformation (Mehmood et al., 2021). The alteration of expression of karyopherins has been linked to the disruption of protein transport, a phenomenon commonly observed in various types of cancer. Over-expression of exportin 1 (XPO1) was observed in many cancers, for example, pancreatic, colorectal, and myeloma. This overexpression can lead to increased cytoplasmic localization and degradation of tumour suppressors and cell cycle-negative regulators, such as p53, RB1, or p21 (Sellin et al., 2022). Similarly, the increased expression of importin subunit α-1 was associated with enhanced cytoplasmic localization of DNA damage response proteins, such as BRCA1, RAD51, or CHK1, in breast cancer (Alshareeda et al., 2015).

Hepatocellular carcinoma (HCC) is the fifth most prevalent cancer type worldwide and the third leading cause of cancer-related deaths (Sung et al., 2021). The prognosis for HCC is generally poor and therapeutic options for advanced stages are limited. Therefore, there is a significant need for the development of new therapeutic strategies. Nucleocytoplasmic transport alterations emerged as a crucial oncogenesis-supporting mechanism in various cancers that could be targeted to impair cancer growth. The role of nucleocytoplasmic transport in HCC is starting to be investigated, and several members of the karyopherin family, such as importin subunit α-1 or exportin 2, have been shown to be over-expressed in HCC (Winkler et al., 2016; Guo et al., 2019).

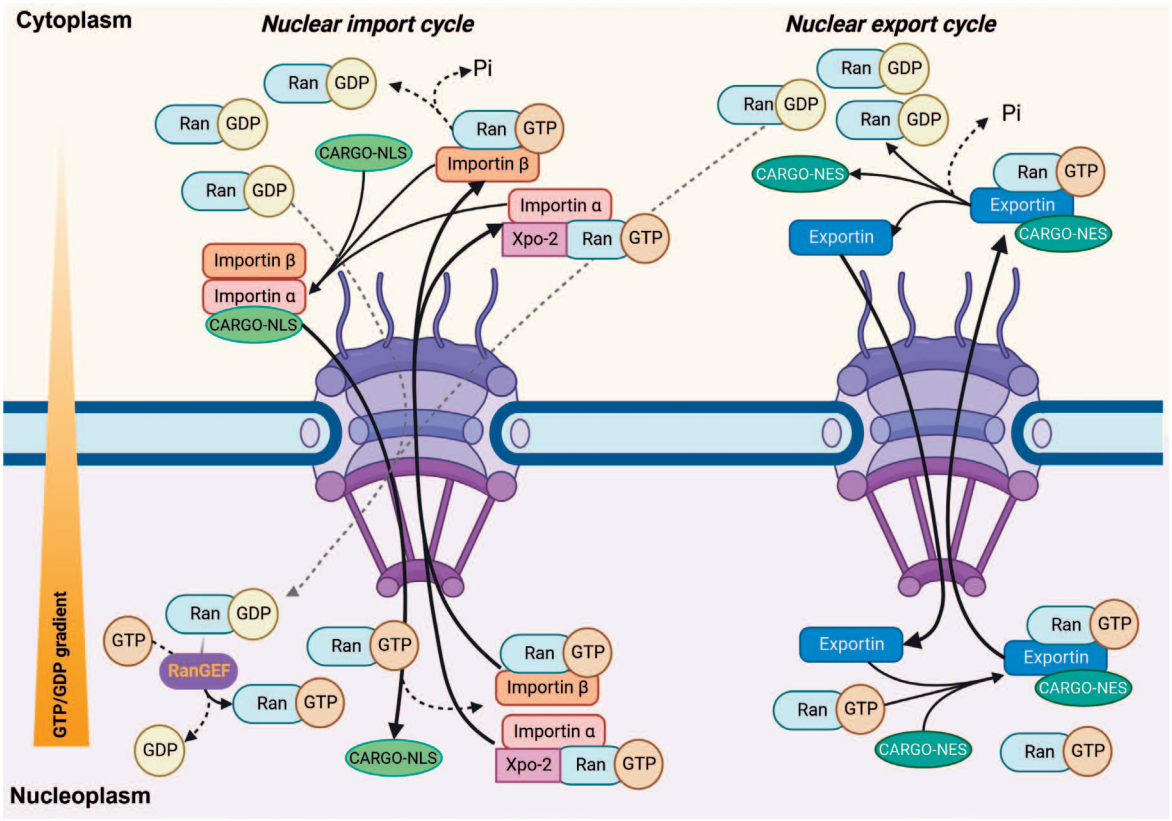

Fig. 1. Graphical representation of the nuclear import and export cycle. (Left) Nuclear import is dependent on importin α and β subunits that bind NLS of the cytoplasmic cargo and are transported into the nucleus, where this tri-complex is dissociated through binding with RanGTP and exportin 2 (XPO2), and importin subunits are recycled back to the cytoplasm. (Right) Nuclear export is facilitated by exportins that bind NES of the nuclear cargo and RanGTP, and this tri-complex is exported to the cytoplasm, where it dissociates after RanGTP hydrolysis. Exportins and RanGDP are translocated back to the nucleus, where RanGDP is loaded with GTP through RanGEF, which ensures maintenance of the RanGTP/RanGDP gradient. Created with BioRender.com.

Therefore, to comprehensively map the role of nucleocytoplasmic transport in HCC, we conducted bioinformatic profiling of expression of nucleocytoplasmic shuttling genes in HCC and their association with clinical outcomes using publicly available datasets. Using genomic, transcriptomic and proteomic data, we have showed that the expression patterns of karyopherin genes are significantly altered in HCC. Moreover, we identified a subset of genes whose expression is most significantly altered in HCC and is associated with worse clinical outcomes. In addition, we have found out that the expression of this subset of genes is associated with pathways promoting cell cycle progression, chromosome segregation and RNA metabolism. Therefore, these genes could be used not only as diagnostic and prognostic markers for HCC but also as targets for the development of novel therapies and/or co-treatment regimens.

Material and Methods

Detection of genetic and expression alterations

To investigate genetic and expression alterations, we employed TCGA (The Cancer Genome Atlas) data using cBioportal (Cerami et al., 2012; Gao et al., 2013). From the available datasets in cBioportal, we chose the latest TCGA Liver Hepatocellular Carcinoma (Firehouse legacy) dataset containing 379 samples. Out of 379 patients in the selected dataset, 360 samples contained data for mutations, copy number alterations (CNA) and mRNA expression scores. Therefore, these 360 liver hepatocellular carcinoma patients' datasets were used for the analysis of genetic alterations as well as expression changes.

Heatmap construction, network and pathway analysis

For heatmap construction, mRNA expression values for 360 HCC were extracted from the TCGA Liver Hepatocellular Carcinoma (Firehouse legacy) dataset and plotted using the SRplot Heatmap module (Tang et al., 2023). For network and pathway analysis, mRNA expression values for 19,660 genes from the TCGA Liver Hepatocellular Carcinoma (Firehouse legacy) dataset were extracted together with Spearman's correlation coefficient with shortlisted karyopherin genes. The top 100 most significantly co-expressed genes with individual karyopherins were analysed using the Cytoscape open-source software that integrates biomolecular interaction networks with high-throughput expression (Shannon et al., 2003). Networks were constructed using a 0.5 confidence (score) cut-off utilizing a full STRING network approach. Constructed interaction networks were curated and exported as png. Subsequently, co-expressed genes were analysed for functional enrichment, and generated enriched pathways from the Gene Ontology (GO) biological process category were plotted using the SRplot Pathway enrichment bubble plot module.

mRNA and protein expression analysis

mRNA expression of individual karyopherins was extracted from the TCGA database, analysed using the TNMplot webtool (Bartha and Győrffy, 2021), and adapted using MS Excel. Targetgrams were constructed in TNMplot from mRNA-seq data from TCGA. Histologically stained HCC tumour and control liver sample images and staining intensity and quantity were extracted from The Human Protein Atlas (https://www.proteinatlas.org/; Uhlen et al., 2017). Antibodies used for individual karyopherins were as follows: importin subunit α-1 – #CAB015460, exportin 2 – #HPA038059, importin subunit β1 – #CAB034449, importin 5 – #HPA040983, importin 7 – #HPA019002, exportin 1 – #HPA042933.

Construction of Kaplan-Meier curves

Kaplan-Meier survival curves were constructed using built-in tools provided by cBioPortal using RNA-seq data from 360 patients with HCC. The plots were generated through patient stratification based on mRNA expression levels as well as gene alterations for individual karyopherins and were generated for each subgroup.

Statistical analysis

The statistical tools were embedded in the resources we used. Briefly, in cBioportal, a two-sided Fisher's exact test was used to determine whether the identified relationship is significant for each gene pair (P value), while examining a tendency of co-occurrences and mutual exclusivity. The q value represents the false discovery rate (FDR) associated with each testing pair (Tables 2, 3). In TNMplot, the Mann-Whitney test was used to determine whether the mRNA expression changes of selected karyopherins were significantly different between

Table 2. Co-occurrence of expression changes for shortlisted karyopherin genes in hepatocellular carcinoma

Gene A	Gene B	P Value	q Value	Tendency
KPNA2	*KPNB1*	< 0.001	< 0.001	Co-occurrence
KPNA2	*CSE1L*	< 0.001	< 0.001	Co-occurrence
KPNB1	*IPO7*	< 0.001	< 0.001	Co-occurrence
IPO5	*IPO7*	< 0.001	< 0.001	Co-occurrence
KPNA2	*IPO7*	< 0.001	< 0.001	Co-occurrence
IPO5	*IPO9*	< 0.001	< 0.001	Co-occurrence
KPNB1	*IPO9*	< 0.001	< 0.001	Co-occurrence
KPNB1	*CSE1L*	< 0.001	0.003	Co-occurrence
KPNA2	*IPO9*	< 0.001	0.009	Co-occurrence
KPNA2	*IPO5*	< 0.001	0.009	Co-occurrence
XPO1	*CSE1L*	0.001	0.015	Co-occurrence
KPNA2	*XPO1*	0.004	0.034	Co-occurrence
KPNB1	*IPO5*	0.005	0.039	Co-occurrence

Table 3. Co-occurrence of HCC driver gene alterations and expression changes for shortlisted karyopherin genes

Gene A	Gene B	P Value	q Value	Tendency
TP53	*CSE1L*	< 0.001	< 0.001	Co-occurrence
TP53	*KPNA2*	< 0.001	< 0.001	Co-occurrence
TP53	*XPO1*	< 0.001	0.002	Co-occurrence
TP53	*IPO5*	0.015	0.033	Co-occurrence
TP53	*IPO9*	0.024	0.047	Co-occurrence
RPS6KA3	*IPO9*	< 0.001	0.002	Co-occurrence
RPS6KA3	*KPNA2*	< 0.001	0.003	Co-occurrence
RPS6KA3	*IPO7*	0.003	0.008	Co-occurrence
RPS6KA3	*IPO5*	0.012	0.027	Co-occurrence
ARID2	*KPNB1*	< 0.001	< 0.001	Co-occurrence
ARID2	*IPO7*	0.001	0.004	Co-occurrence
ARID2	*IPO5*	0.004	0.010	Co-occurrence
FGFR4	*CSE1L*	< 0.001	< 0.001	Co-occurrence
ARID1A	*IPO9*	0.007	0.018	Co-occurrence

HCC and normal liver samples. For Kaplan-Meier curve statistical analysis, a log-rank test was used to determine whether the difference in overall survival between selected patients' cohorts was significant. In Cytoscape, for pathway enrichment analysis, FDR was calculated using the Benjamini-Hochberg procedure.

Results

Genetic alterations and gene expression of karyopherins are significantly altered in HCC

To comprehensively investigate the genetic and transcriptomic alterations of all 26 karyopherin genes present in the human genome (Table 1) in liver cancer, we analysed the TCGA genomic and transcriptomic data for 366 patients with HCC. Our analysis shows that genes for karyopherins are altered in 46 % of samples, amplification being the most prevalent gene alteration (Fig. 2A, B). The alteration frequency of individual genes varied from 0.5 % for *KPNA1* to 10 % for *IPO9*. The majority of genes that were altered were amplified, and the only gene that was deleted in a larger number of samples was *XPO7* deleted in 8 % of samples (Fig. 2A, B). Although there were several point mutations present in multiple karyopherin genes, these mutations were present in a minuscule fraction of samples, which shows that single-nucleotide mutations in karyopherin genes do not represent cancer-promoting alterations.

On the other hand, expression changes larger than 2-fold in both directions were much more prevalent in HCC (70 % samples) (Fig. 2C, D). The majority of expression changes were up-regulations, with *IPO9* being the most up-regulated gene in striking 31 % of samples, and most down-regulated genes were *XPO7* and *KNPA6*; however, the expression down-regulation was not uniform across all the samples (Fig. 2C, D). Next, we performed unsupervised clustering of karyopherin expression changes in HCC samples and identified two clusters with significantly increased expression – cluster I containing *KPNA2*, *IPO9*, and *CSE1L* and cluster II containing *KPNB1, IPO5, IPO7*, and *XPO1* (Fig. 3A). This prompted us to further investigate the expression of these seven karyopherins in HCC compared to non-transformed liver tissue. We found that mRNA expression of all shortlisted karyopherins was significantly increased in HCC compared to normal liver tissue with various fold changes (Fig. 3B, C, D). In general, the expression of cluster I karyopherins was higher (range 1.92–4.02 fold increase) (Fig. 3B, C) than cluster II karyopherins (range 1.43–1.98) (Fig. 3B, D), *KPNA2* and *CSE1L* having the highest expression increase of 4.02 and 2.4, respectively (Fig. 3B). Additionally, we compared the gene expression of individual karyopherins among each other within the same HCC sample, as well as in respect to known HCC driver alterations (*TERT, TP53, CTNNB1, AXIN1, ARID1A, RPS6KA3, FGFR4*) (Ding et al., 2017) to identify any synergies between the alteration of nucleocytoplasmic shuttling and HCC driver mutations. As shown in Table 2, *KPNA2* and *KPNB1* were significantly co-over-expressed with each other as well as with other members of both clusters, especially with importins *IPO5*, *IPO7*, and *IPO9*, indicating functional association to enhance nuclear export of various cargoes. Additionally, we compared the expression of shortlisted karyopherin genes with genetic alterations driving HCC. We have shown that mutations in *TP53*, *RPS6KA3*, and *ARID2* are significantly correlated with increased expression of each of the shortlisted karyopherin genes and *KPNA2*, *IPO9,* and *IPO5* showed most consistent co-occurrence with those mutations, which further strengthens the link between HCC and karyopherin expression (Table 3).

Protein expression of a subset of karyopherins is significantly increased in HCC in comparison to non-transformed tissue

To further strengthen the link between HCC and karyopherin expression, we analysed histological staining of karyopherins in samples of HCC and normal liver tissues obtained from the Human Protein Atlas database (https://www.proteinatlas.org/). In agreement with mRNA expression data, we observed over-expression of the majority of shortlisted karyopherins in HCC samples compared to the normal liver (Fig. 4A–D). We observed strong over-expression of importin subunit α-1 (*KPNA2*), importin subunit β-1 (*KPNB1*), importin 5 (*IPO5*), and exportin 1 (*XPO1*), whereas expression of exportin 2 (*CSE1L*) and importin 7 (*IPO7*) was increased only moderately in comparison to normal liver tissue (Fig. 4A–D). Importin subunit α-1, importin subunit β-1, importin 5, and exportin 1 showed strong nuclear staining, while expression of importin 7 was mainly seen in the cytoplasm in both normal liver and HCC samples (Fig. 4A–D).

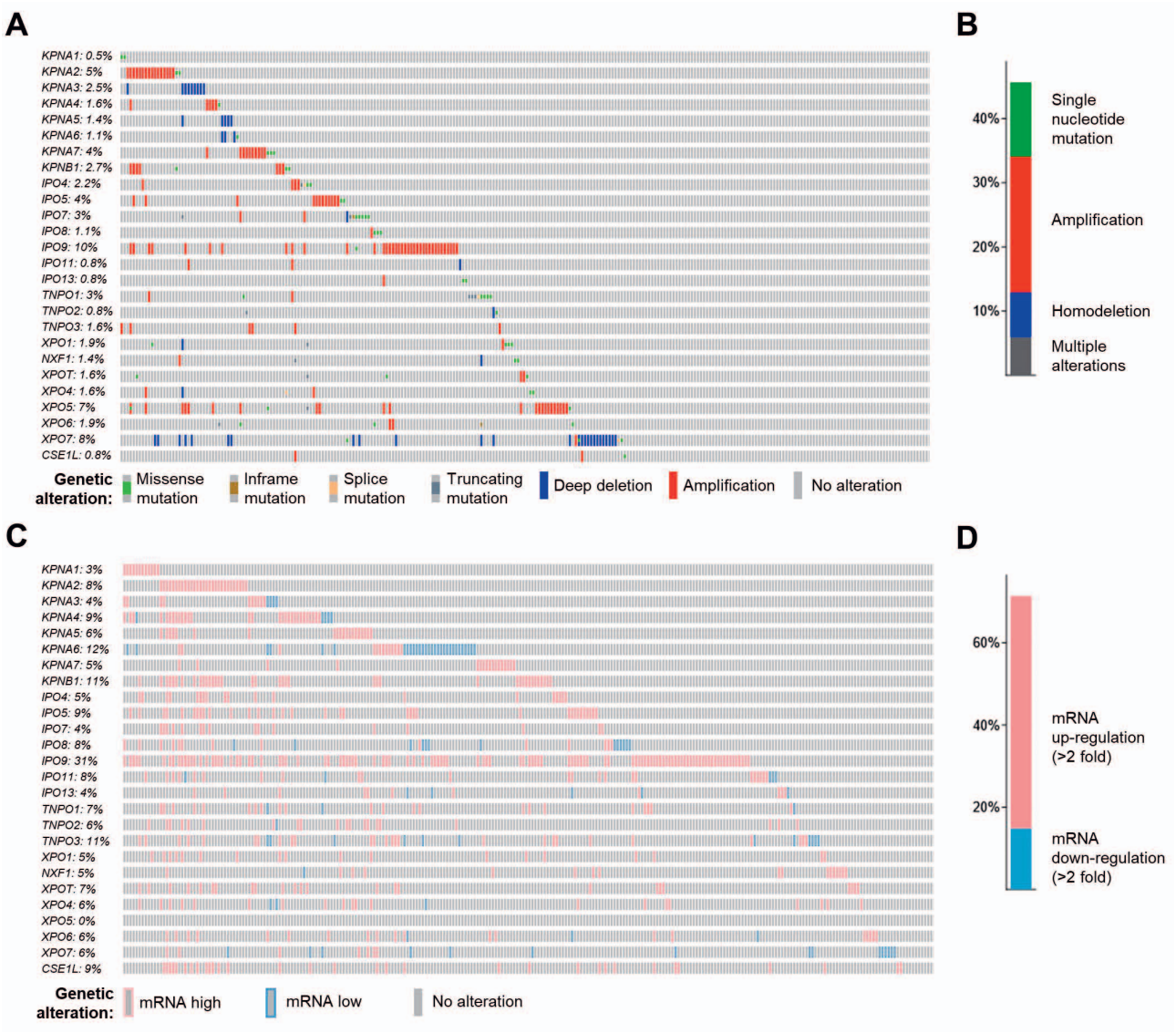

Fig. 2. Genomic and transcriptomic alteration of karyopherin genes in HCC: (**A**, **B**) The frequency of various genetic alterations in HCC samples. The majority of the alterations are gene amplifications followed by single-nucleotide mutations and homodeletions. (**C**, **D**) The frequency of gene expression alteration (fold change > 2) is more prevalent in HCC, and ~50 % of samples show a significant increase in the expression of karyopherin genes.

Over-expression of a subset of karyopherins is negatively correlated with patients' overall survival

To analyse the prognostic value of genetic alteration and expression of karyopherins for HCC patients, we investigated the association of karyopherin expression and overall survival using the TCGA data. We analysed the prognostic value of karyopherin genes both individually and as clusters (I: [*KPNA2*, *IPO9*, *CSE1L*]; II: [*KPNB1, IPO5, IPO7, XPO1*]). The expression of cluster I genes was significantly increased in 37 % of patient samples and was negatively correlated with the HCC patients' overall survival, with a median survival of 27 months for the high expression cohort compared to 80 months of the low expression cohort (Fig. 5A, E). Moreover, the expression of cluster I genes was also inversely correlated with the histological grade of HCC tumours (Fig. 5F). When looking at individual genes, the expression of *KPNA2* was associated with the worst clinical outcome, with median survival for the high expression cohort of only 14 months compared to the low expression for the cohort of 70 months (Fig. 5B). On the other hand, the expression of *IPO9* was less detrimental to the HCC patients' survival; however, it was over-expressed (> 2-fold change) in the highest proportion of samples – 31 % (Fig. 5C). Interestingly, genetic alterations of karyopherin genes were not associated with changes in the HCC patients' survival, indicating that expression is the determining factor of karyopherin functionality in HCC (Supplementary Fig. 1A–D). The expression of cluster II

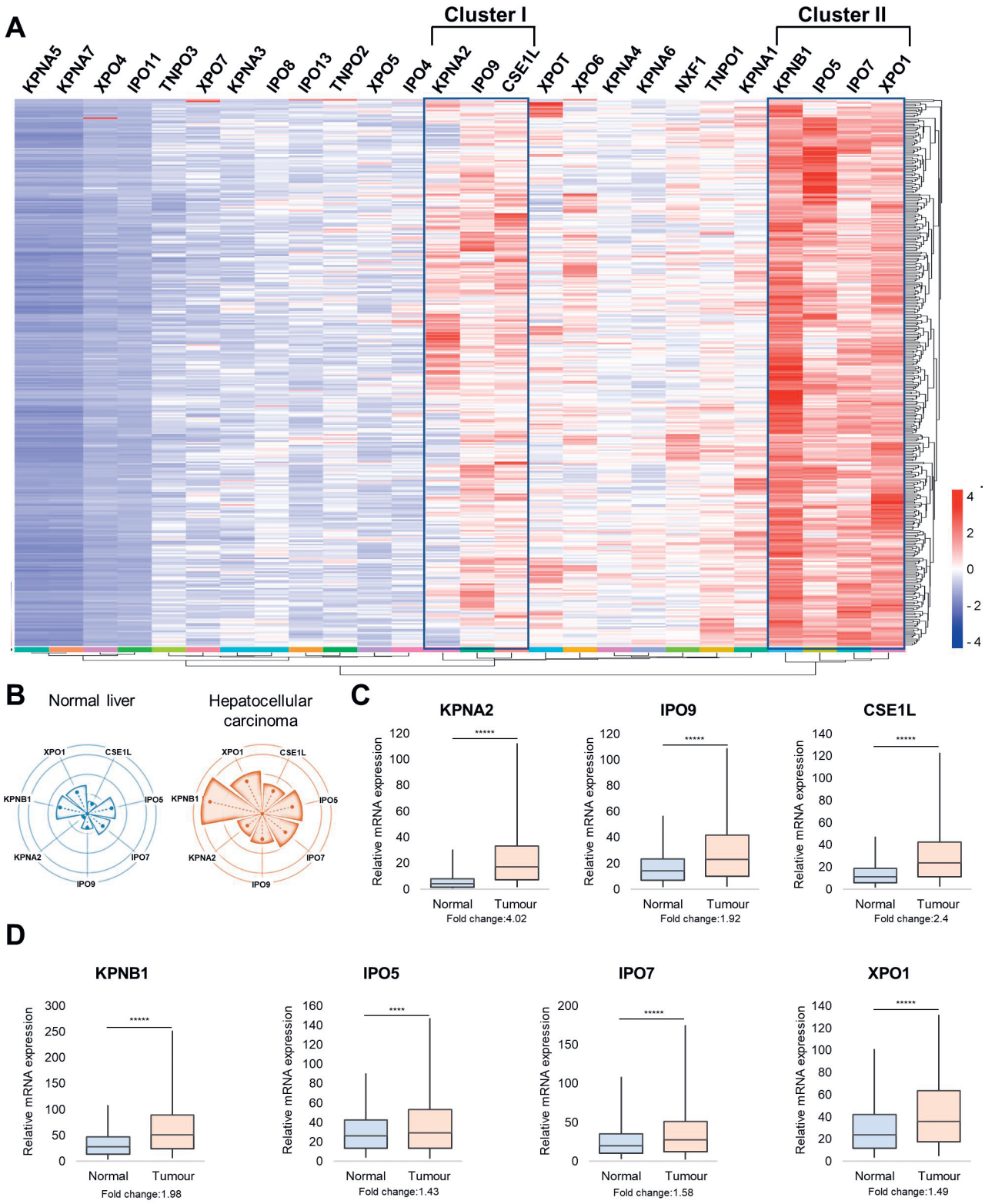

Fig. 3. mRNA expression of karyopherin genes in HCC and normal liver tissue. (**A**) Heatmap of mRNA expression for individual karyopherin cluster karyopherin genes based on the expression pattern. Red colour signifies increased mRNA expression, blue colour signifies decreased mRNA expression. (**B**) The targetgram shows an overview of mRNA expression for a selected gene set in the normal liver (left) and HCC (right). The size of the segments represents the mean values; the length of the dashed lines represents the median values of each type. (**C**, **D**) The box plot shows mRNA expression of selected karyopherins in samples of the normal liver (N = 225) and HCC (N = 371). Statistical significance is calculated using the Mann-Whitney method; $^{****}P < 0.0001$, $^{*****}P < 0.00001$.

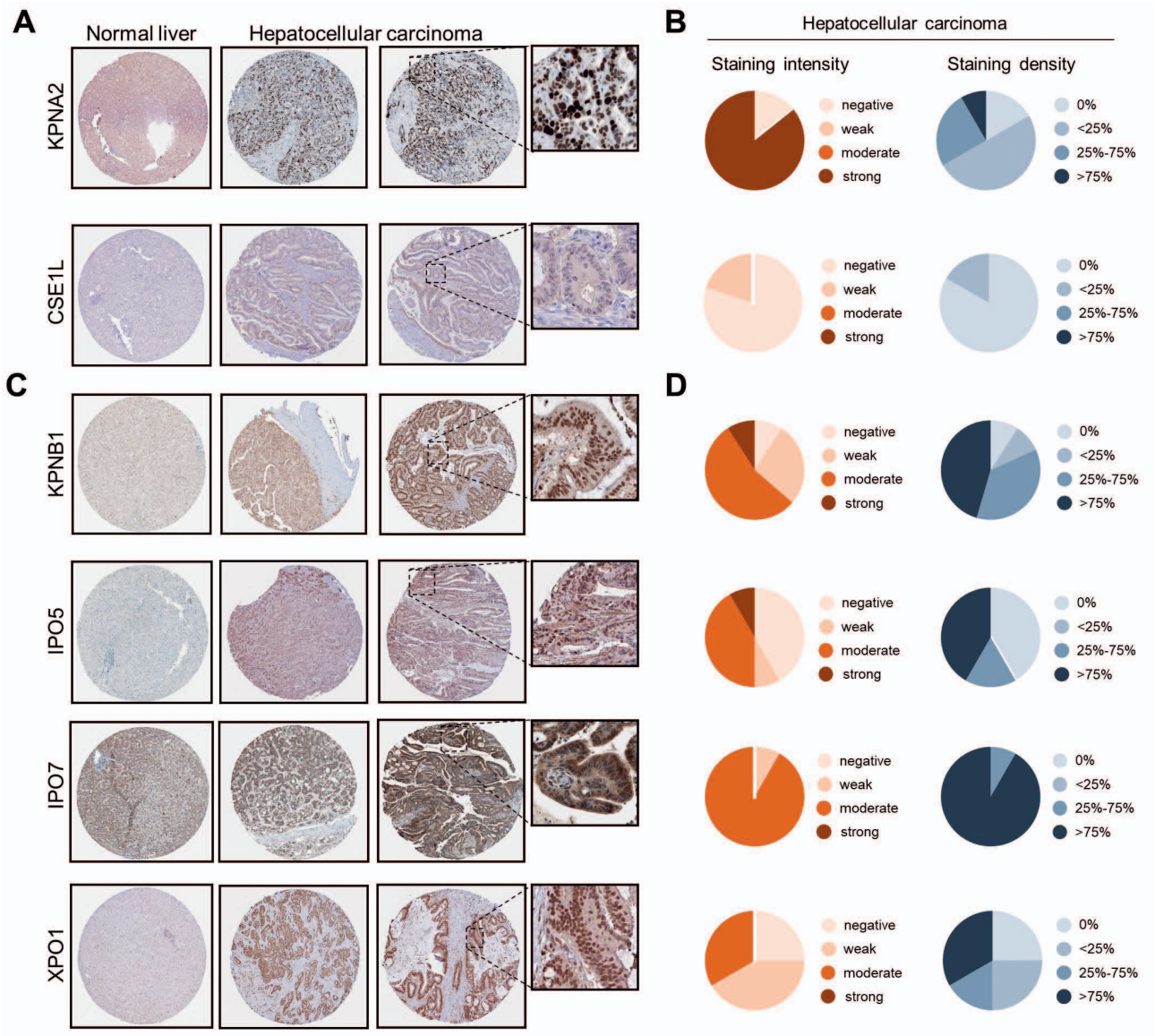

Fig. 4. Histological analysis of expression of shortlisted karyopherin genes in HCC. (**A**, **C**) Needle biopsy cores of normal liver tissue (left panel) and HCC (right panel) were stained using specific antibodies against individual karyopherins. (**B**, **D**) Histology staining was scored according to pathology guidelines and plotted to represent staining intensity and density for each individual karyopherin. Images available from v23.proteinatlas.org.

genes was significantly increased in 20 % patients, and it was also negatively correlated with the HCC patients' overall survival (high expression cohort median survival – 25 months, low expression cohort median survival – 70 months) (Fig. 6A, F). Moreover, the expression of cluster II karyopherin genes was again significantly correlated with a higher histologic grade of HCC neoplasms (Fig. 6G**)**. At the level of individual genes, the increased expression of *KPNB1* was the major predictor of shorter overall survival in HCC patients (high expression cohort median survival – 14 months, low expression cohort median survival – 70 months) (Fig. 6B). Similarly, the expression of *IPO7* and *XPO1* was significantly inversely correlated with a worse overall patient survival; however, the portion of samples with a > 2-fold expression increase was 4 % in case of *IPO7* and 6 % in case of *XPO1* (Fig. 6D–F). On the other hand, the expression of *IPO5* was not significantly correlated with a decreased overall patient survival (Fig. 6C); however, reanalysis of the same dataset with lowering the cut-off to 1.5-fold increase showed that indeed, the increased expression of *IPO5* is significantly correlated with a worse patient survival (high expression cohort median survival – 33.2 months, low expression cohort median survival – 58.84 months) (Supplementary Fig. 2D). Similar to cluster I karyopherin genes, genetic alterations in cluster II genes were not associated with changes in the clinical outcomes of HCC patients (Supplementary Fig. 2A–F).

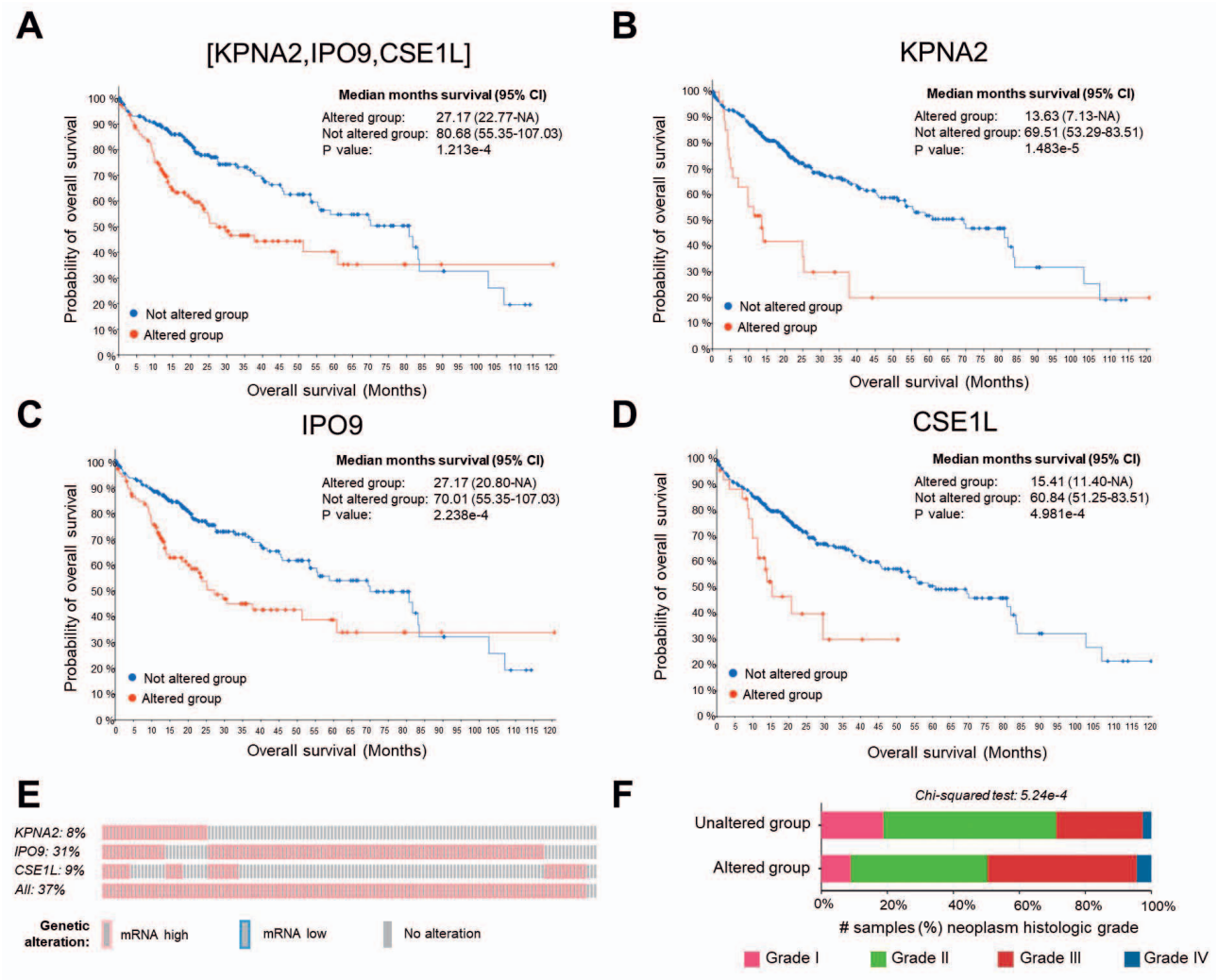

Fig. 5. Association of mRNA expression of shortlisted karyopherin genes with clinical outcomes of HCC patients. (**A–D**) Kaplan-Meier plots showing overall survival; the X-axis shows months after the diagnosis, the Y-axis shows overall survival probability. Patient cohorts were split based on the mRNA expression for the respective karyopherin (fold change > 2); the blue line indicates the non-altered patient cohort, the red line indicates the altered patient cohort. The table shows the median months of survival for both cohorts; P and q values were calculated using the Log-rank test. (**E**) Frequency of gene expression alteration (fold change > 2) in samples from HCC patients. (**F**) Relative frequency of patients from high and low expression cohorts (split by 2-increase in mRNA expression for cluster I karyopherins combined) that were diagnosed with specific neoplasm histological grade.

Over-expressed karyopherins regulate key pathways implicated in HCC pathophysiology

Finally, to characterize the pathways that are potentially regulated by shortlisted karyopherins in HCC, we analysed the genes that are significantly co-expressed with the individual shortlisted karyopherins. We extracted the co-expression data from TCGA and performed pathway enrichment analysis for the top 100 most significantly positively correlated genes with each of the shortlisted karyopherins. Our analysis showed that cluster I karyopherins (*KPNA2, IPO9, CSE1L*) are significantly co-expressed with genes regulating cell cycle progression, and especially the progression of mitosis, including spindle organization and chromosome separation (Fig. 7A–C). Especially *KPNA2* and *CSE1L* showed the strongest association with pathways regulating mitotic progression, and these proteins formed a very tight functional interaction network containing most of the correlated genes with short nodes (Fig. 7A, C, Supplementary Fig. 3A, B). The *IPO9* expression was also highly correlated with the expression of genes regulating cell cycle progression; however, additional enriched pathways were identified, including regulation of RNA localization, DNA damage response, or histone modification, indicating the broader role of *IPO9* (Fig. 7B, Supplementary Fig. 3C). For cluster II karyopherins, the functional pathway enrichment was more variable among

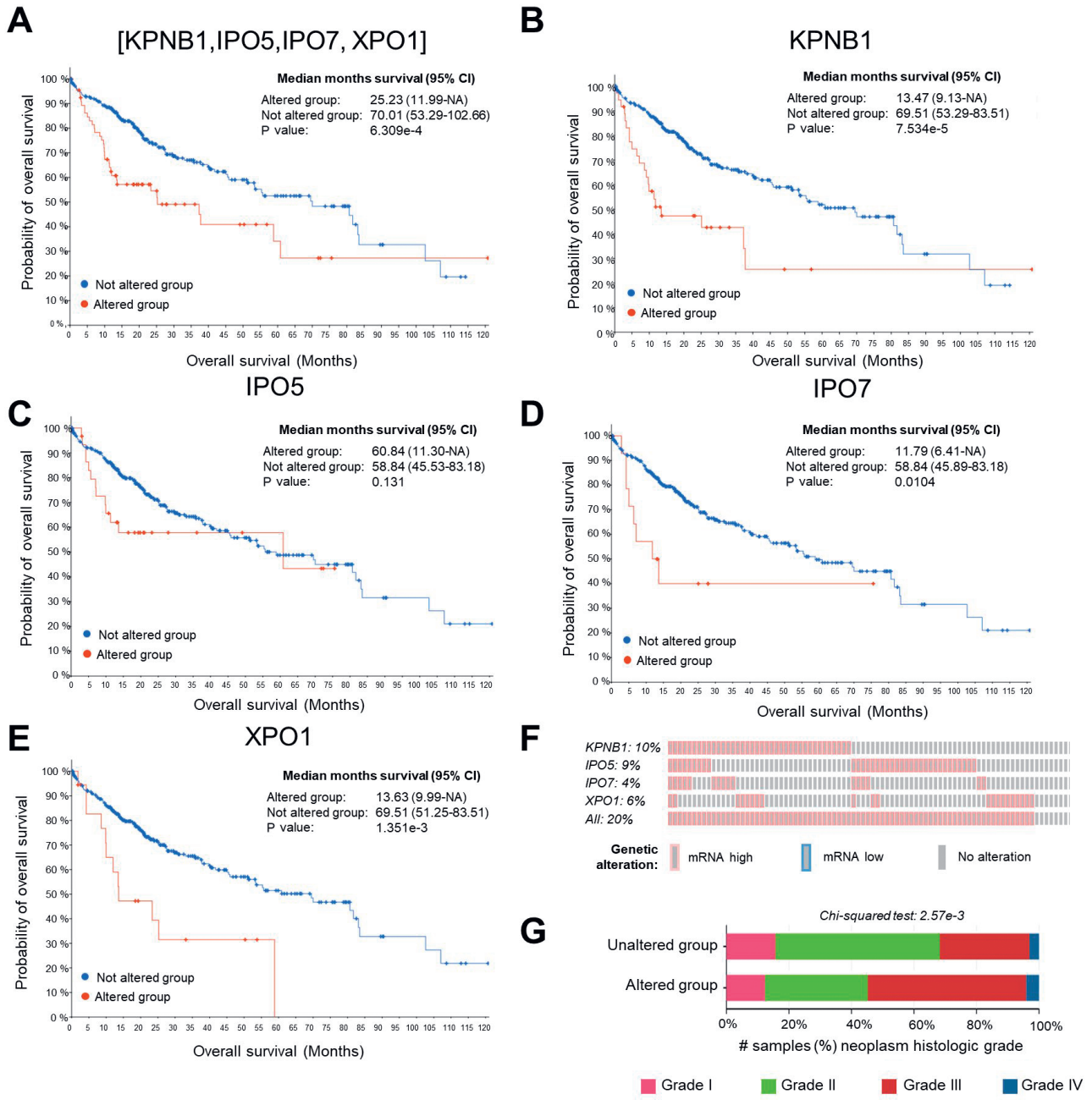

Fig. 6. Association of mRNA expression of shortlisted karyopherin genes with clinical outcomes of HCC patients. (**A–E**) Kaplan-Meier plots showing overall survival; the X-axis shows months after the diagnosis, the Y-axis shows overall survival probability. Patient cohorts were split based on the mRNA expression for the respective karyopherin (fold change > 2); the blue line indicates the non-altered patient cohort, the red line indicates the altered patient cohort. The table shows the median months of survival for both cohorts; P and q values were calculated using the Log-rank test. (**F**) Frequency of gene expression alteration (fold change > 2) in samples from HCC patients. (**G**) Relative frequency of patients from high and low expression cohorts (split by 2-increase in mRNA expression for cluster I karyopherins combined) that were diagnosed with specific neoplasm histological grade.

the members. Similarly to *KPNA2* and *CSE1L, KPNB1* was most significantly associated with the regulation of cell cycle progression, mitosis, and chromosome segregation but also with other pathways such as histone modification and DNA damage repair, and this broader functionality is reflected by a looser network containing fewer correlated genes with longer nodes grouped into several functional interaction clusters (Fig. 7D, Supplementary Fig. 3D). The remaining cluster II karyopherins were associated with several different pathways, which are reflected by much less tight interaction networks (Supplementary Fig. 4). *IPO5* was strongly associated

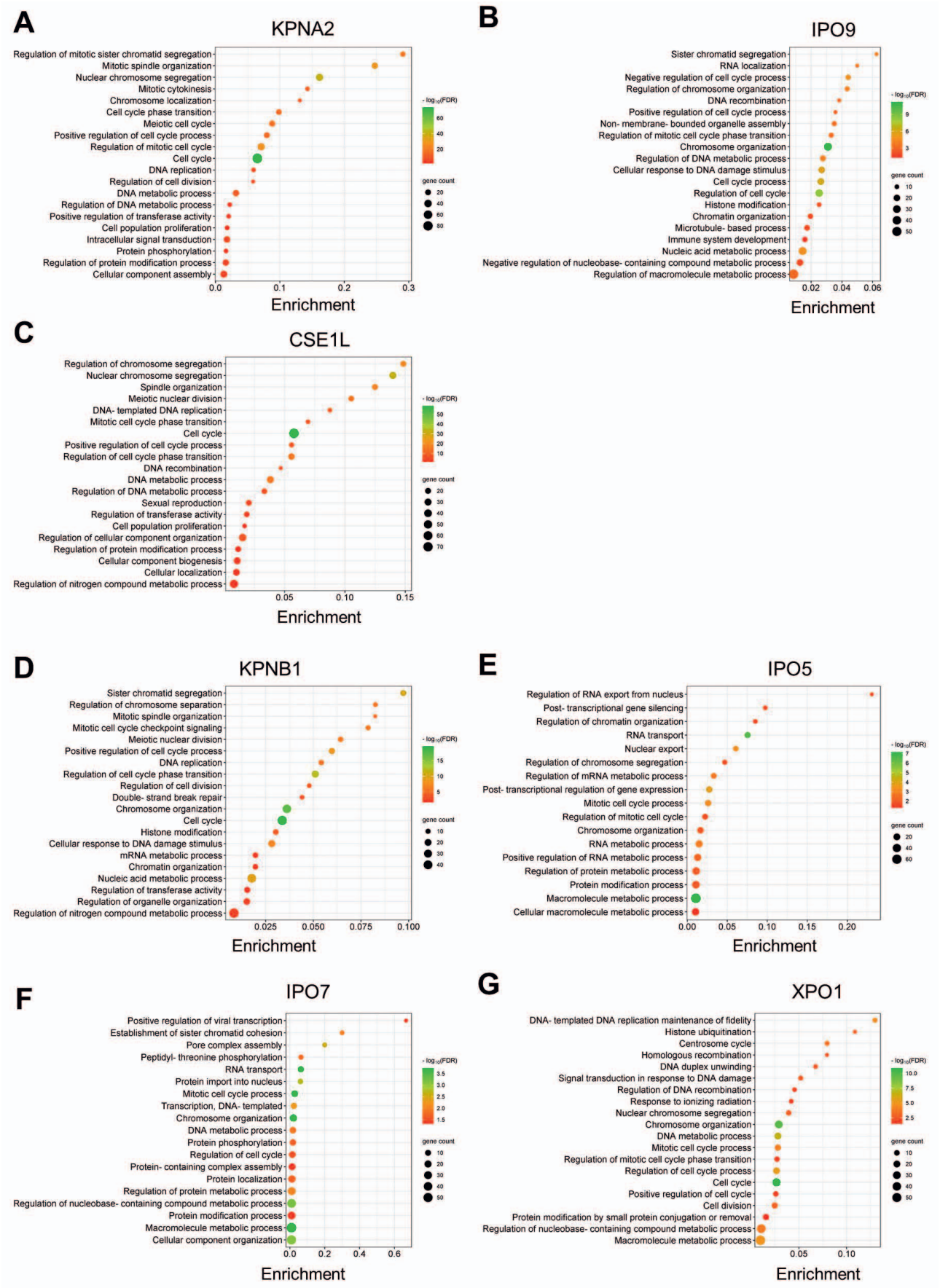

Fig. 7. Pathway enrichment analysis of the top 100 positively correlated genes with shortlisted karyopherins. (**A–G**) Bubble charts showing the top 20 enriched GO biological process pathways constructed from the top 100 genes most significantly co-expressed with individual karyopherins. Pathways are sorted based on the gene enrichment score. The bubble size indicates the number of co-expressed genes enriched in the particular pathway. The colour indicates a negative logarithm of false discovery rate (FDR), green indicates low FDR, red indicates higher FDR.

with pathways regulating RNA export and metabolism (Fig. 7E). The *IPO7* expression showed strong association with pathways regulating nucleocytoplasmic shuttling – pore complex assembly, RNA transport, protein nuclear import, and others (Fig. 7F). The *XPO1* expression was strongly associated with DNA damage repair processes as well as cell cycle progression regulation (Fig. 7G). Finally, the identified pathways enriched in genes co-expressed with selected karyopherin genes are in agreement with already identified respective cargo proteins (Supplementary Table 1).

Discussion

Although nucleocytoplasmic transport plays an indispensable role in regulating normal cellular physiology, it has received only a limited amount of scientific interest in regard of its role in oncogenesis. Proteins from the family of karyopherins play an essential role in mediating the transport of molecules in and out of the nucleus, thus regulating key processes such as gene expression, DNA damage, cell cycle progression, or RNA metabolism. It is well established that deregulation of the above-mentioned processes contributes to oncogenesis. Karyopherins transport various cellular proteins, and there are cargoes specific to a certain karyopherin but also cargoes that can be bound by several different transport proteins. Moreover, in the case of importins, the complexes consist of α and β subunits, and the α subunit further interacts with another member of the karyopherin family – exportin 2, which mediates their recycling from the nucleus to the cytoplasm. Because of this extensive interaction network between individual karyopherins, we set out to examine the molecular alterations of the entire karyopherin family in HCC, which has limited systemic treatment options.

Our analysis shows that there is a significant degree of genomic and transcriptomic alteration of karyopherin genes; however, genomic alterations are less prevalent than expression changes. Overall, genomic alterations in karyopherin genes were present in 45 % of the HCC samples, and mRNA expression of karyopherin genes was altered in 71 % (> 2-fold cut-off) of HCC samples and reached up to 90 % with a less stringent cut-off (> 1.5-fold change). There are very few single-nucleotide changes in karyopherin genes and there is no hotspot mutation, indicating that there is no driver mutation that would be crucial for HCC development. On the contrary, multiple karyopherin genes are amplified, and this alteration is the most prevalent in *IPO9* (10 % of patients) and *KPNA2* (5 % of patients). Interestingly, there is only one gene that was significantly deleted in HCC patients – *XPO7* (8 % of patients). On the other hand, alterations of mRNA expression were much more prevalent in the samples from HCC, and roughly 70 % of samples showed more than 2-fold expression changes in HCC compared to normal liver tissue. Similarly to genetic alterations, the expression of *IPO9* (31 % of patients) and *KPNA2* (8 % of patients) was significantly up-regulated. Additionally, the expression of *CSE1L* (9 % of patients), *KPNB1* (11 % of patients), and *IPO5* (9 % of patients) was uniformly significantly up-regulated in HCC. Combined analysis of karyopherin expression identified seven genes grouped into two clusters whose expression was significantly up-regulated in HCC in comparison to the normal liver, namely *KPNA2, IPO9, CSE1L* (cluster I), and *KPNB1, IPO5, IPO7*, and *XPO1* (cluster II). Interestingly, all of these proteins except exportin 1 facilitate the nuclear import of various cargoes. Importin 9, as the most up-regulated karyopherin, is responsible for importing histones as well as proteasomal subunits, which indicates that it plays a crucial role in DNA replication and cell cycle progression (Padavannil et al., 2019; De Almeida et al., 2021). Similarly, importin subunit α-1 (*KPNA2)* cargoes include DNA damage repair proteins such as BRCA1, NBS1, RAD51, or cell cycle regulator E2F1 (Alshareeda et al., 2015; Drucker et al., 2019), suggesting an important role in the regulation of DNA damage response as another hallmark of cancer. Finally, exportin 2 (*CSE1L*) is a crucial factor in importin recycling, thus stimulating the import of different pro-cell cycle proteins (Jiang, 2016). Cargoes of importin subunit β-1 and importin 7 cargoes, encoded by the cluster II genes, *KPNB1* and *IPO7*, include multiple transcription factors and cell cycle-promoting proteins, such as cyclin B1, SREPB2, CREB, ERK2, or SMAD3 that play a key role in promoting cell proliferation (Mackmull et al., 2017). Moreover, importin 7 imports histone proteins as well as ribosomal proteins RPL5 or RPS7 (Dean et al., 2001). Similarly to importin 7 and importin 9, importin 5 is responsible for importing histone proteins as well as ribosomal proteins, which further strengthens its role in promoting cell cycle progression (Jakel, 1998).

Interestingly, the only protein facilitating the export of various cargoes that was significantly up-regulated is exportin 1 (*XPO1*), which is a well-studied gene with established pro-oncogenic function (Nguyen et al., 2012). Increased mRNA expression is also translated into increased protein expression, although not uniformly across all the analysed karyopherins. Finally, importin subunit α-1, importin subunit β-1, importin 5 and exportin 1 protein expression (immunohistochemistry staining) was significantly up-regulated in tissue samples from HCC compared to the normal liver, and these proteins could be useful as diagnostic and possibly also prognostic markers.

Another key conclusion that could be drawn from the analysis is that the expression of karyopherins is positively correlated with higher HCC neoplasm histological grade and negatively correlated with the patients' overall survival. The increased expression of *KPNA2, CSE1L, KPNB1, IPO7*, and *XPO1* was strongly associated with a shorter patients' survival, where the median month survival for patients with increased expression (> 2-fold) was only around 13 months, whereas the median month survival for patients without increased expression was around 70 months. This correlates with the

up-regulation of the cell cycle and mitosis-promoting pathways that are enriched in genes that are co-expressed with karyopherins associated with worse patient prognosis. Interestingly, we did not identify any such correlations between genomic alterations of individual karyopherin genes and patient survival. This further strengthens the notion that expression changes rather than mutations in karyopherin genes are crucial oncogenesis-promoting events.

Even though the expression of individual karyopherins is associated with higher histology grade and worse prognosis, it is crucial to understand the interactions between individual karyopherins because the transport system is greatly interconnected. α and β importins interact together with cargo proteins and are transported in the nucleus. Therefore, the relative abundance of different types of importins could lead to qualitative as well as quantitative changes in imported cargoes that would enable oncogenic signalling. This is supported by the fact that increased expression of multiple individual karyopherins co-occurs in the same HCC samples. Moreover, there is a significant association of karyopherin expression with several genomic alterations in established HCC driver genes, such as *p53*, *S6KA3*, or *ARID2*, further indicating a crucial role of nucleocytoplasmic shuttling alterations in HCC molecular oncology.

Our analysis shows that nucleocytoplasmic transport is significantly altered in HCC, associated with worse HCC patients' outcomes, and highly likely contributes to pro-oncogenic signalling. Given the limited treatment options for advanced HCC, nucleocytoplasmic transport represents a promising target for the development of novel therapeutics. At the moment, there are inhibitors of several karyopherins such as exportin 1, importin subunit β-1, or importin subunit α-1 being tested mostly *in vitro*; however, some of them are also in the later stages of development. For example, inhibition of importin subunit β-1 by compound DD1-Br showed anti-tumour activity in the mouse models of castration-resistant prostate cancer (Kelenis et al., 2022). Moreover, exportin 1 inhibitor Selinexor is now approved by FDA for treatment of multiple myeloma and is being investigated as monotherapy or in combination with conventional anticancer treatment for other haematological as well as various solid tumours such as breast, endometrial, ovarian, or lung cancer (Nachmias and Schimmer, 2020; Landes et al., 2023; Mo et al., 2023). Despite encouraging results in other cancer types, the utility of these inhibitors is severely underexplored in HCC (reviewed in Wing et al. (2022)). Taken together, our work indicates that the nucleocytoplasmic shuttling system is a viable target for developing novel therapeutic regimens for the treatment of hepatocellular carcinoma.

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