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Research article

ALTERATIONS OF THE Hsp70/Hsp90 CHAPERONE AND THE HOP/CHIP CO-CHAPERONE SYSTEM IN CANCER

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Abstract: Activation of the Hsp90 chaperone system is a characteristic of cancer cells. The regulation of chaperone activities involves their interaction with co-chaperones; therefore we investigated the expression of Hsp70 and Hsp90 and their specific co-chaperones HOP and CHIP in cancer cell lines and primary cancers. Inhibition of Hsp90 by 17AAG increased the levels of Hsp70, Hsp90 and HOP but not CHIP mRNA in cancer cells. These changes are linked to activation of the HSF1 transcription factor and we show that the HOP promoter contains HSF1 binding sites, and that HSF1 binding to the HOP promoter is increased following 17AAG. The lack of alteration in the co-chaperone CHIP is explained by a lack of HSF response elements in the CHIP promoter. Non-proliferating cells expressed higher levels of CHIP and lower HOP, Hsp70 and Hsp90 levels compared to proliferating cells. Decreased expression of CHIP in proliferating cancer cells is in keeping with its proposed tumor suppressor properties, while over-expression of HOP in proliferating cells may contribute to excessive Hsp90 activity and stabilization of client proteins in tumors. In a panel of colorectal cancer samples, increased expression of Hsp70 and an increased ratio of HOP to CHIP were found, and were associated with decreased median survival. These data indicate that multiple changes occur in the chaperone/co-chaperone system in cancer that impact patient survival. It is likely that the ability to identify individual alterations to this system will be beneficial for treatment strategy decisions, particularly those that employ chaperone inhibitors.

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Abbreviations used: CHIP – C terminus of Hsc70-interacting protein; HOP – Hsp70/Hsp90 organizing protein; HSE – heat shock element; HSF1 – heat shock factor 1; Hsp70 – heat shock protein 70; Hsp90 – heat shock protein 90; qRT-PCR – quantitative real-time polymerase chain reaction; 17AAG – 17-(allylamino)-17-demethoxygeldanamycin

Key words: Chaperone, Co-chaperone, Cancer, Hsp90, Hsp70, HOP, CHIP, HSF1, 17AAG

INTRODUCTION

During the multi-step process of carcinogenesis, malignant transformation and genetic instability processes produce mutated or unstable proteins which, along with overexpressed oncogenes, have to be folded and stabilized by the chaperone system. Therefore, transformed cells are dependent on increased chaperone activity and this was accordingly recognized as a hallmark characteristic of cancer cells [1].

Within the chaperone system, the cooperation of Hsp70 and Hsp90 is essential for effective folding of numerous proteins related to cancer progression, and the involvement of Hsp90 in proliferation and survival-related signaling pathways makes cancer cells dependent on its activity. Therefore, the inhibition of Hsp90 by small molecules represents a unique approach for cancer therapy by targeting multiple oncogenic processes simultaneously [1]. The efficacy of Hsp90 inhibitors in cancer treatment is ascribed to a higher affinity of tumor Hsp90 to its inhibitors [2]. However, the exact mechanism(s) responsible for hyper-activation of Hsp90 and its increased sensitivity to inhibitors is not understood. One possible explanation is that increased activity of Hsp90 in tumors results from global changes in chaperones and co-chaperones that participate in the Hsp90 folding cycle. The aberrant expression of co-chaperones and their altered interaction with Hsp90 could explain the increased ATPase activity of Hsp90 and its high affinity for inhibitors.

Within the Hsp70/Hsp90 system, the co-chaperone HOP (Hsp70/Hsp90 organizing protein, also called STIP1, Stress-Inducible Protein 1) facilitates chaperoning activity of Hsp90 *via* simultaneous binding to both Hsp70 and Hsp90 to mediate the transfer of a client protein from Hsp70 to Hsp90 [3]. It has been observed that HOP is over-expressed in some cancers and is thought to have pro-tumorigenic properties [4-6]. Conversely, the co-chaperone CHIP (C terminus of Hsc70-interacting protein) is a chaperone-associated E3 ubiquitin ligase which targets unfolded proteins for degradation, and its down-regulation has been associated with advanced cancer and higher grade [7]. Both HOP and CHIP bind to the C-termini of Hsp70 and Hsp90, and thus compete with each other for binding to chaperones [8]. Due to the opposite impacts of HOP and CHIP on chaperone activity, we hypothesized that their altered expression is an important factor for regulating the overall activity of chaperones in cancer cells. To test this hypothesis, we analyzed the expression of CHIP, HOP, Hsp70 and Hsp90, and we demonstrate that HOP is regulated by the HSF1 (heat shock factor 1) transcription factor that is known to regulate Hsp70 and Hsp90 [9], indicating coordinated regulation of these components of the pro-folding chaperone system in cancer cells. We also analyzed the expression levels of

Hsp70, Hsp90, HOP and CHIP in colorectal cancer samples to correlate the constitution of the chaperone system with cancer prognosis.

MATERIALS AND METHODS

Cell culture and treatment

Human cancer cell lines HCT116 and H1299 were cultured in D-MEM, 10% fetal bovine serum and pyruvate. Cells were grown to 60-80% confluence before experimental treatment. 17AAG (17-(allylamino)-17-demethoxygeldanamycin) was added to a final concentration of 4 μM and cells were exposed for 8 hours. Cell transfections were performed by Amaxa (Lonza) according to the manufacturer's instructions. siRNA was purchased from Dharmacon, Thermo. Cell line H1299-175 was developed in our laboratory [10].

SDS-PAGE and immunoblotting

Cells were washed twice in ice-cold PBS and lysed in buffer containing 0.5% CHAPS, 150 mM NaCl, 50 mM HEPES pH 7.5 and complete protease inhibitor cocktail (Sigma-Aldrich). A measure of 10 μg of total protein was separated by SDS–polyacrylamide gel electrophoresis (SDS–PAGE) in 10% gels and transferred onto nitrocellulose membranes (Bio-Rad). The blotted membranes were blocked in 5% milk and 0.1% Tween 20 in PBS for 1 h at room temperature and probed overnight with specific monoclonal antibodies. The antibodies used in this study were as follows: antibody recognizing HOP (Moravian Biotechnology), antibody Pab1801 recognizing p53 [11], HSF1 (Santa Cruz Biotechnology) and AC-40 monoclonal antibody which recognizes actin (Sigma-Aldrich). Peroxidase-conjugated rabbit anti-mouse immunoglobulin antiserum or swine anti-rabbit immunoglobulin antiserum (Dako) was used as the secondary antibody. To visualize peroxidase activity, ECL reagents (Amersham Pharmacia Biotech) were used according to the manufacturer's instructions.

Chromatin immunoprecipitation

Cells were cross-linked with 1% formaldehyde for 10 min at 37ºC. The reaction was stopped with 1 M glycine. After sonication, DNA bound to proteins was immunoprecipitated with HSF1 antibody (Santa Cruz Biotechnology) or beta galactosidase antibody BG2. The purified DNA was used for qRT-PCR to amplify the region -1324 to -1063 upstream of initiation codon of the HOP gene using the following primers: forward 5´-ccatagccccagtaggacaa-3´ and reverse 5´-aacaacctgcagggtcagtc-3´.

Tissue sampling

50 colon cancer patients were enrolled in this study at the Masaryk Memorial Cancer Institute. Ethical permission was granted after review at the Masaryk Memorial Cancer Institute and all patients gave written informed consent. Samples of the colonic tumor tissue were received within 20 minutes of surgical

removal according to the standardized hospital protocol and immediately evaluated by a pathologist. Tissue samples were frozen and stored in liquid nitrogen.

Reverse transcription and qRT-PCR

Total cellular RNA from colorectal tissue samples was extracted using TRI Reagent (Ambion) and RNA from cell lines was extracted using RNeasy Mini Kit (Qiagen). cDNA synthesis was carried out using random hexamer primers, dNTPs, RiboLock RNase Inhibitor and RevertAid H Minus Reverse Transcriptase (all Fermentas) following the manufacturer's protocol. RNA samples were not subjected to a denaturation step. Triplicate samples were subjected to quantitative PCR analysis using SYBR Green Master (Roche) and 7900HT Fast Real-Time PCR system (Applied Biosystems). The primer pairs used for qRT-PCR were Hsp90α_F 5′-tctggctgagggatgacttac-3′, Hsp90α_R 5′-acgttaccccaatctgtgaaa-3′, Hsp90β_F 5′-agcctcgagtgcccctgtc-3′, Hsp90β_R 5′-catccaatcctgctgtcaagagta-3′, Hsp70_F 5′-cgtgctcatctttgacctgg-3′, Hsp70_R 5′-gggttgatgctcttrttcag-3′, CHIP_F 5′-gagaatggctgggtggaggactac-3′, CHIP_R 5′-ctcagcccagcccaccctcacct-3′, HOP_F 5′-ccccaggcactcagcgaacact-3′, HOP_R 5′-cagcggcggcacaaacagc-3′, ActB_F 5′-gccgacaggatgcagaaggag-3′, ActB_R 5′-ctagaagcatttgcggtggac-3′. Absolute mRNA expression levels obtained using the standard curve method were normalized to the levels of β-actin.

Statistical analysis of data

Patients were divided into two groups according to the expression levels of a particular gene (low and high expression). Survival rates were plotted against time using the Kaplan-Meier method and log-rank testing was used to compare the differences between the curves. Statistical significance was defined as $p < 0.05$. Data were analyzed using GraphPad Prism 5.03 software.

RESULTS

The regulation of expression of Hsp70, Hsp90, HOP and CHIP in cancer cell lines

We treated five different cancer cell lines with a specific small molecule inhibitor of Hsp90, 17AAG, which causes dissociation of HSF1 from Hsp90 and leads to trimerization and activation of HSF1 [12], and measured the mRNA levels of Hsp70, Hsp90α, Hsp90β, HOP and CHIP by qRT-PCR. The expression of Hsp70 was analyzed by primers detecting all major cytoplasmic isoforms of Hsp70. We found that all analyzed genes except CHIP were consistently up-regulated by 17AAG treatment (Fig. 1). We also used serum starvation to test whether the induction of cell cycle arrest influences the expression levels of chaperones and co-chaperones. Fig. 2 shows that the expression of HOP, Hsp70, Hsp90α and Hsp90β are down-regulated in non-proliferating serum-starved cells in contrast to CHIP.

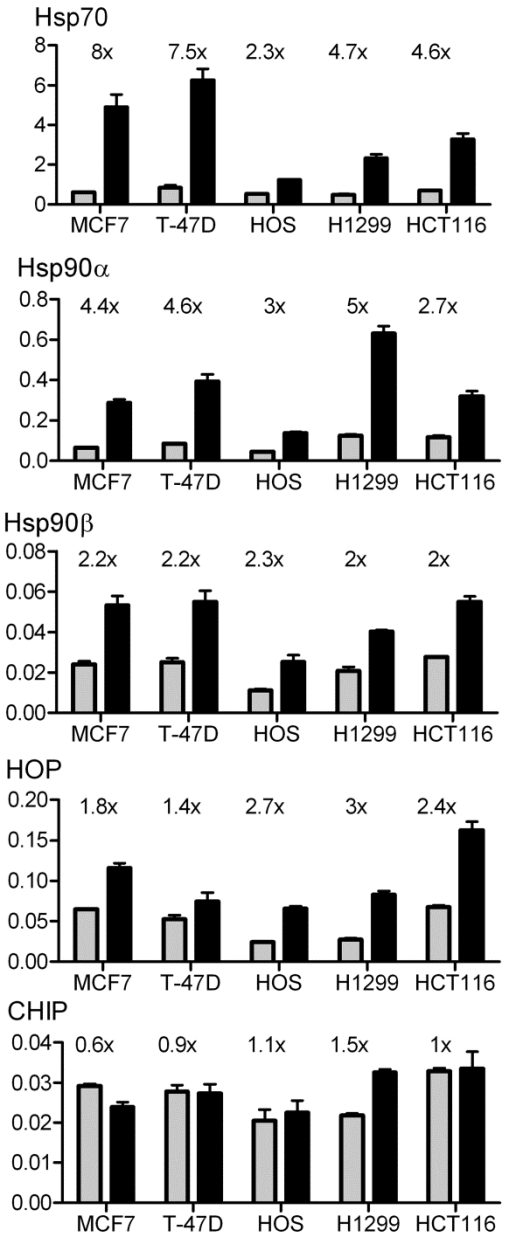

Fig. 1. Effect of 17AAG treatment on the transcription of chaperones and co-chaperones. Graphs show mRNA levels of Hsp70, Hsp90α, Hsp90β, HOP and CHIP normalized to the actin levels. Gray columns correspond to the control cell lines and black to the 17AAG treated cells. Numbers represent fold induction of mRNA levels.

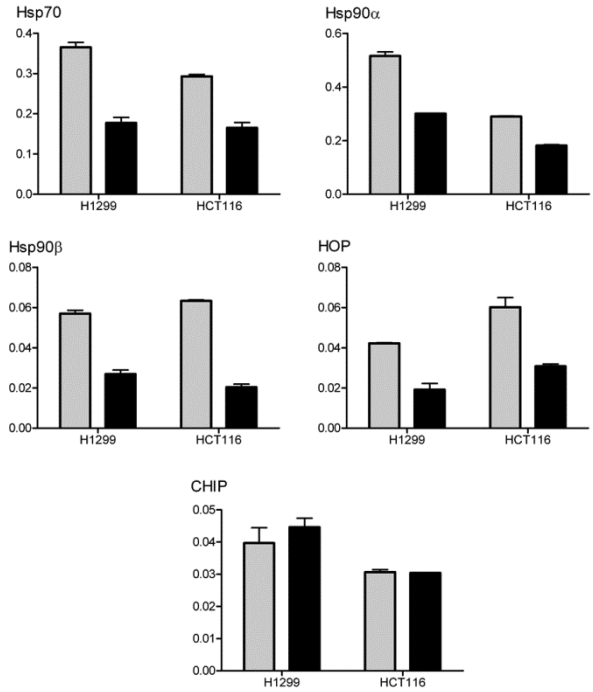

Fig. 2. Effect of serum starvation on the expression levels of chaperone machine. Graphs show mRNA levels of chaperones and co-chaperones in the cell lines H1299 and HCT116. mRNA levels of analyzed genes were normalized to the actin levels. Control cells (represented by gray columns) were cultivated in the full medium and starving cells (black columns) were cultivated in the medium without fetal bovine serum.

The expression of HOP is directly regulated by HSF1

The above data indicate that the expression of the co-chaperone HOP is regulated in a similar manner to the Hsp70 and Hsp90 chaperones. It is well known that these two chaperones are under the regulation of the HSF1 transcription factor, but whether HSF1 also regulates HOP is not known. To investigate whether expression of HOP is regulated by HSF1, we analyzed the HOP promoter for HSE elements and used chromatin immunoprecipitation for HSF1. There are two complete HSE (heat shock element) elements (GAANNTTCNNGAA) within the HOP promoter, in opposite orientations -260 and -680 base pairs from the transcription start site (Fig. 3A). Chromatin immunoprecipitation confirmed binding of HSF1 to the HOP promoter and showed increased HSF1 binding after Hsp90 inhibition by 17AAG (Fig. 3B and C).

The influence of chaperone gene expression on survival of patients with colorectal cancer

To investigate the clinical significance of altered chaperone/co-chaperone expression we measured the expression of Hsp70, Hsp90α, Hsp90β, HOP and CHIP in a panel of 50 colorectal cancer samples and correlated their relative

expression with survival. Absolute copy numbers of mRNAs were obtained using the standard curve method and these values were normalized to the levels of β-actin. Patients were divided into two groups according to the expression levels of each analyzed gene and subjected to statistical analysis of overall survival (Fig. 4 and Table 1). Patients with low levels of Hsp70 survived for more than twice as long as patients with high Hsp70 levels (median survival 1281 or 542 days, respectively). Levels of both Hsp90α and Hsp90β also influenced survival. Expression levels of HOP or CHIP did not affect median survival on their own. However, we identified an important effect of the ratio between their expression levels; median survival of patients expressing lower HOP/CHIP ratio was 1257 days compared to 580 days for patients with a high HOP/CHIP ratio, supporting the importance of functional competition between HOP and CHIP.

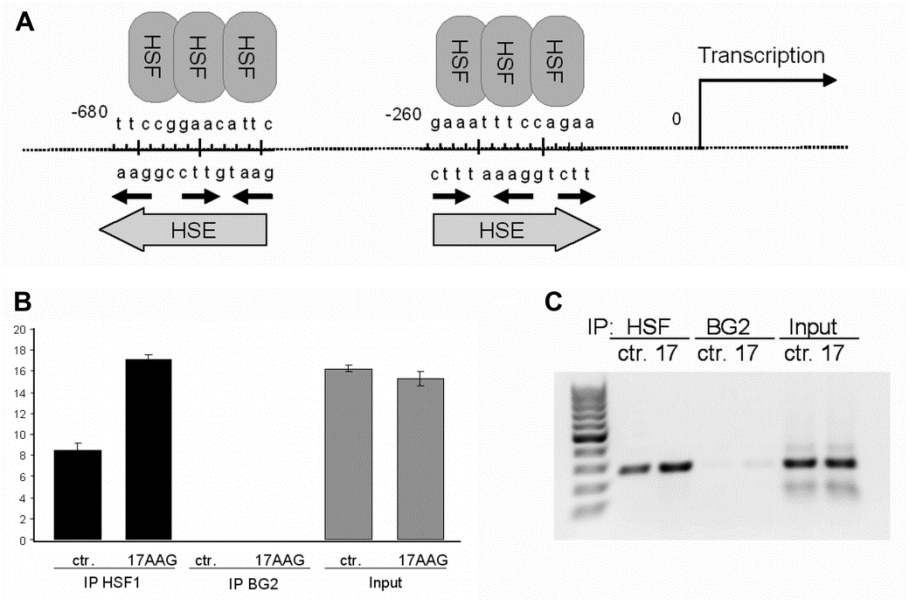

Fig. 3. Chromatin immunoprecipitation assay (ChIP). A – HOP promoter contains two HSE elements -260 and -680 base pairs from the transcription start. B – The binding of HSF1 to the HOP promoter was estimated in 293 cells either untreated or exposed to 17AAG. Transcription factor HSF1 was precipitated using anti-HSF1 antibody. Anti-beta galactosidase antibody was used as a negative control. Promoter DNA was then detected by qRT-PCR. Graph shows absolute copy numbers obtained using standard curve method. C – Agarose gel electrophoresis of ChIP products amplified by PCR.

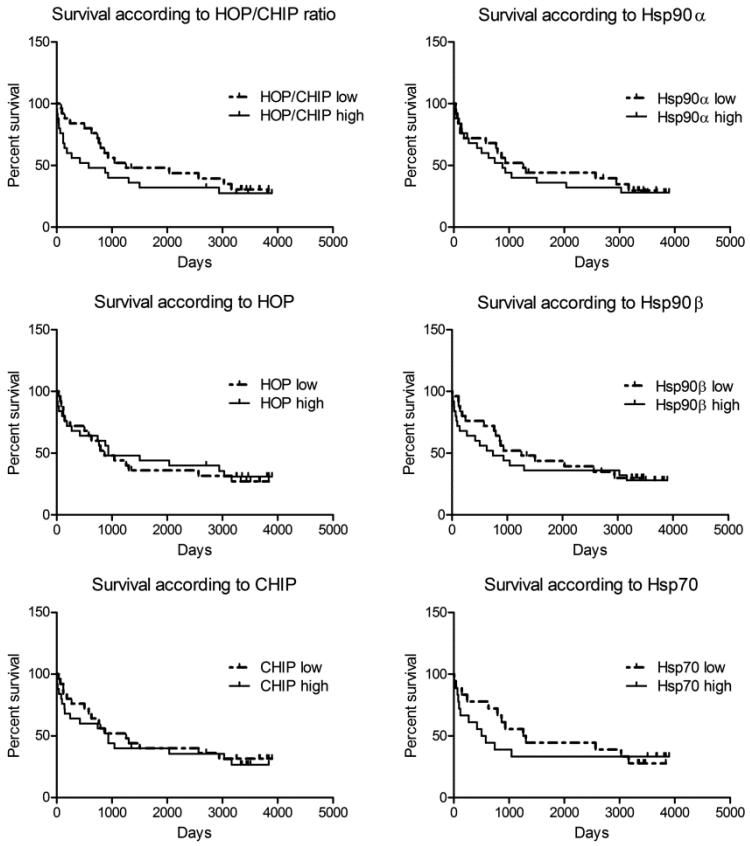

Fig. 4. Kaplan-Meier survival analysis. Survival curves of patients with colorectal carcinoma according to the expression levels of co-chaperones HOP, CHIP and their ratio and chaperones Hsp90α, Hsp90β and Hsp70. The continuous curves represent the group of tumors with high expression of individual genes and the dashed curves represent the group with low expression.

Table 1. Median survival of patients. Median survival of patients grouped according to the expression levels of Hsp70, Hsp90α, Hsp90β, HOP, CHIP and HOP/CHIP ratio is expressed in days.

Gene	Median survival in days		Log-rank (Mantel-Cox) test	
	Low levels	High levels	Chi square	P-value
Hsp90α	1257	878	0.1443	0.7041
Hsp90β	1257	740	0.3479	0.5553
Hsp70	1281	542	0.2058	0.6501
HOP/CHIP	1257	580	1.041	0.3075
HOP	865	933	0.0747	0.7845
CHIP	1257	931	0.248	0.6185

The correlation between Hsp70 and HOP/CHIP ratio

The analysis of chaperone expression in colorectal cancers showed that the expression levels of Hsp70 and the HOP/CHIP ratio had the most significant impacts on patient survival. We also assessed potential correlations between levels of Hsp70, HOP and CHIP and demonstrated that Hsp70 levels correlate with the HOP/CHIP ratio (r=0.59 p= 0.001, Fig. 5A). This correlation is mainly due to an inverse relationship between Hsp70 and CHIP (Fig. 5B), rather than a direct correlation of Hsp70 with HOP (Fig. 5C).

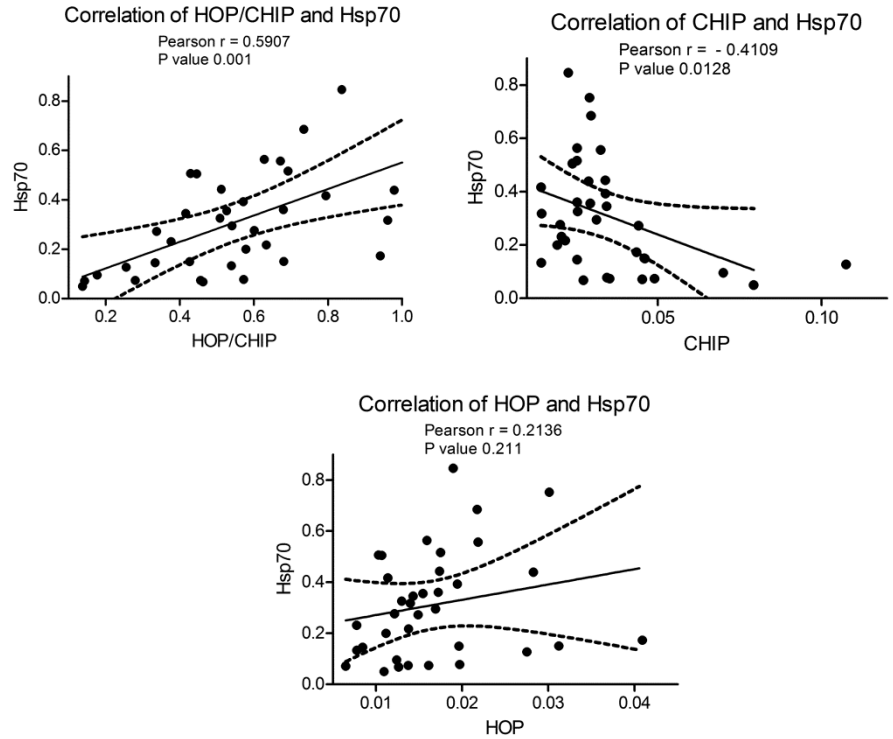

Fig. 5. Correlation analysis. mRNA levels of HOP, CHIP and HOP/CHIP ratio from colorectal tumors revealed distinct relations with Hsp70 mRNA levels. Graphs show Pearson correlation analysis of each combination of genes with indicated r and P values. Graphs comprise regression line with 95% confidence interval.

The overexpression of HOP activates HSF1

The data above indicate coordinated regulation of the chaperone/co-chaperone system in cancer cells involving up-regulation of Hsp70, Hsp90 and HOP and/or down-regulation of CHIP. We have also demonstrated that HSF1 regulates HOP expression, in addition to its well-known effects on chaperone expression. To investigate whether HOP is directly involved in the coordinated regulation of this system, we manipulated the expression of HOP and tested the levels of HSF1 and of Hsp70. Inhibition of HOP expression by siRNA caused a reduction of HSP70 mRNA and of HSF1 protein levels, whereas overexpression of HOP

resulted in slightly increased expression of Hsp70 and HSF1 (Fig. 6). The effect of HOP on protein folding was also investigated by measuring the levels of R175H mutant p53, which requires Hsp90 activity for stabilization [10]. These data demonstrate that mutant p53 stability is decreased by a lack of HOP and increased by HOP over-expression, in keeping with HOP providing a protein folding function.

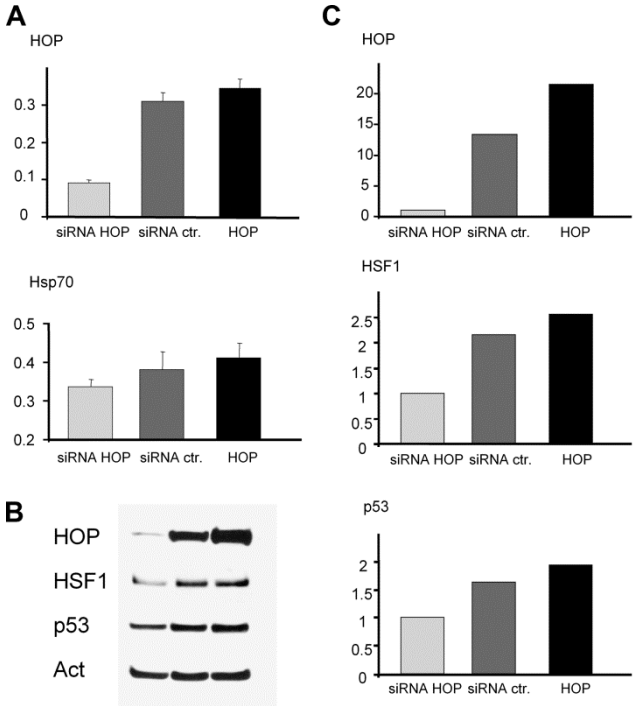

Fig. 6. Modulation of HOP levels in the cell line H1299 R175H. HOP levels were either downregulated by siRNA or upregulated by transfection of recombinant HOP. A – Absolute mRNA levels of HOP and Hsp70 normalized to the levels of actin. B – Protein levels of HOP, HSF1, p53 and actin were then determined by western blotting. C – Graphs show relative protein levels quantified using TotalLab.

DISCUSSION

Chaperone overexpression has been observed in many cancer types previously and has a negative impact on prognosis [13-15]. Several recent studies also described increased expression of the co-chaperone HOP in specific cancers [4-6] and have suggested a down-regulation of CHIP [7]. Our study confirmed a decrease in overall survival of patients with higher levels of Hsp90α, Hsp90β and Hsp70 with the strongest effect of Hsp70. We also observed an impact of the HOP/CHIP ratio on survival rates in contrast to separate analysis of HOP or CHIP levels alone, suggesting the existence of a tight regulatory circuit for the chaperone machinery involving interactions with co-chaperones. It is known that

the co-chaperones HOP and CHIP interact with the same binding motif of both Hsp90 and Hsp70 and the nature of these interactions is competitive [16]. This implies that the character of the chaperone machinery activity (folding or degradation) would be directed by the more abundant co-chaperone. Since HSF1 activation has been linked with cancer [17, 18], our demonstration that HOP is a transcriptional target of HSF1 provides one mechanism by which the chaperone system is pushed towards a dominant protein folding activity by increasing HOP without influencing CHIP expression. In addition, because HSF1 will also induce Hsp70 and Hsp90, this novel regulation of HOP implies that the pro-folding environment can be coordinately induced by HSF1 in cancer. Moreover, that HOP expression itself modulates the activity of the chaperone machinery indicates a further complexity in this regulatory circuit and indicates the existence of positive-feedback loops that further enhance the protein folding environment of cancer cells.

Co-chaperones HOP and CHIP have antagonistic effects on the processing of client proteins in the chaperone machine and compete for binding sites at chaperones Hsp70 and Hsp90 [16]. We therefore suppose that excess of a given co-chaperone drives the chaperone machine activity either to folding or degradation of client proteins. The master regulator of the heat shock response, HSF1, is also an Hsp90 client and is held by Hsp90 in the inactive form [19]. The higher pro-folding activity of the chaperone machine may therefore also lead to the elevation of HSF1 levels and subsequent transcription of Hsps and HOP.

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