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# HSP47 A NOVEL COLLAGEN BINDING SERPIN CHAPERONE, AUTOANTIGEN AND THERAPEUTIC TARGET

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## 1.ABSTRACT

Hsp47 was originally discovered as a cell surface collagen binding protein, colligin, and was later shown to be an endoplasmic reticulum (ER) resident protein with collagen binding properties in chick fibroblasts. Hsp47 has been termed J6, gp46, CB48 and CBP2 in various other organisms and has been mapped to human chromosome 11q13.5 a known "hot spot" in a number of human cancers. Hsp47 has been shown to be constitutively expressed with collagens; it is heat inducible and binds to both helical and non-helical forms of collagens. Hsp47 binds closely to procollagen in the ER, but dissociates from it in the *cis*-Golgi to allow fibril formation. Hsp47 is over-expressed in many fibrotic diseases including: glomerulosclerosis, pulmonary fibrosis, liver cirrhosis, cicatricial pemphigoid, epidermolysis bullosa acquista and keloids. Hsp47 is associated with fibrosis following myocardial infarction and has been localized in artherosclerotic arteries. Among a number of rheumatoid conditions, Hsp47 manifests properties of an autoantigen and in some cancers appears to be a biomarker. The unique properties of Hsp47 in modulating collagen production and its location to the cell membrane in many forms of cancer have designated Hsp47 as a potential biomarker and/or therapeutic target for a number of conditions and diseases.

## 2. INTRODUCTION

Hsp47 is a highly conserved protein being found from Leptogorgia virgulata (1) to zebrafish (2-7) to Homo sapiens (8). However, Hsp47 was first described as a collagen binding protein (colligin) isolated from murine parietal endoderm cells (9). Although generally localized to the endoplasmic reticulum (ER), colligin was first shown to interact with the type IV collagen via a cell surface-association. Originally, it was not known what proportion of the colligin (Hsp47) molecules in the cell were associated with the surface, but it was speculated that if colligin (Hsp47) was involved in the processing, glycosylation, and secretion of collagen, a certain proportion might be expected to reach the surface as a result of fusion of vesicles from the Golgi. Another supposition was that colligin (Hsp47) molecules are associated primarily with the cell surface and are required for "spinning out" or cross-linking the three dimensional assembly of type IV collagen molecules (9, 10).However, there is still controversy regarding the collective functions of this novel member of the serpin family. Interestingly, these initial reports had little impact at the time as homologous proteins were described in a number of different organisms over the next few years. For example, colligin was subsequently characterized in humans and rats as gp46 (11-21) , in chickens as Hsp47 (22), CB48 in bovine endothelial cells (23) and in mice as J6 (11-13, 24-27). Later, a human homolog of these proteins, CBP2, was reported from human fetal lung (8), and was mapped to chromosome 11q13.5, which is a "hot spot" in a number of forms of cancer (28-30). To minimize confusion, we will restrict our reference to this protein in all organisms as Hsp47.

## 3. MECHANISMS OF HSP47 GENE REGULATION

In chick fibroblasts, Hsp47 was identified as a ER glycoprotein with major collagen-binding properties that was heat-inducible (6). This gene in mice spans about 7.8 kb, consisting of six exons separated by five introns. The promoter region contains a TATA box, four Sp1-binding sites and one AP-1-binding site. A complete heat-shock element (HSE) was found between nucleotides (nt) -61 and -79.The mouse promoter of the Hsp47 gene also

contains sequence motifs similar to retinoic acid-responsive elements (RAREs) identified in other genes (31, 32). Also, it appears that at least three sequences are required for the constitutive expression of Hsp47 in BALB/c 3T3 cells: the -210 bp Sp1 binding site, the BS5-B element in the first intron, and the EP7-D element in the second intron (31, 33). Moreover, it has been suggested that KLF proteins regulate the transcription of Hsp47 by binding the BS5-B element in cooperation with Sp2 and/or Sp3(34).

Three alternate spliced Hsp47 mRNAs, differing only in their 5' non-coding regions, have been detected in the mouse. However, one of the spliced variants is expressed only after heat shock (35, 36). Other forms of stress, such as azetidine or sodium arsenite, also induce Hsp47 expression, but not similar splicing patterns. The alternatively spliced variant of the heat induced form of Hsp47 is translated more efficiently at elevated temperatures than the constitutive forms, suggesting that alternative splicing may be an important post-transcriptional mechanism of Hsp47 regulation(36). Although, Hsp47 can also be induced by cold shock (1, 37) and is influenced by hypergravity in slow skeletal muscles, the extent of alternative splicing has not been elucidated (38).

## 4. HSP47 PROTEIN STRUCTURE AND FUNCTION

Hsp47 is translated with a signal peptide, which after cleavage results in a 400 amino acid protein possessing two N-linked carbohydrate attachment sites (12). These sites are glycosylated with high-mannose oligosaccharides (39). In addition, the protein contains a C-terminal RDEL ER-retention signal, which if deleted results in secretion of the mutant Hsp47 protein (40, 41). While Hsp47 contains a number of potential phosphorylation sites in various cells, no functional significance has yet to be determined for these phosphorylated forms (17, 18, 42, 43). Hsp47 shares homology with the serpin family of serine protease inhibitors, but it is not active as a protease inhibitor in serine protease *in vitro* assays (44). This is probably because of sequence differences at the active site (45). However, recently Hsp47 has been shown to possibly function as a cross-class inhibitor of cysteine proteinases by a mechanism in which cysteine proteinases assault a peptide bond in the reactive loop of serpins, adjacent to the $P_1$-$P'_1$ bonds involved in serine proteinase inhibition (46). However, whether this reaction has any biological significance *in vivo* has yet to be determined. Interestingly, Hsp47 possesses ~45% homology with the squamous cell carcinoma (SCC) antigens, which belong to the superfamily of serpins and also behave as cross- class inhibitors (47, 48).

## 5. ASSOCIATION OF HSP47 WITH COLLAGEN PRODUCTION

The expression of Hsp47 in various cell lines and tissues has always been closely linked with the expression of various types of collagens under non-stressed conditions (42, 45, 49, 50). The synthesis of type I collagen has been shown to decrease after malignant transformation, this decrease being coincident with that of other extracellular matrix proteins, including fibronectin and laminin (42, 51). Conversely, Hsp47 is dramatically induced during differentiation of teratocarcinoma cells following treatment with retinoic acid (52-54) and parallel induction of Hsp47 with collagens is also reported after treatment of myoblastic cells with transforming growth factor beta-1(54). Hsp47 is also associated with collagen-XVIII (55), and a newly discovered collagen-XXVI (56). The constitutive expression of Hsp47 is always accompanied by that of collagen under non-stressed conditions, however, Hsp47 synthesis is not observed in cells in which collagen synthesis is not observed, such as macrophages, lymphocytes, pheochromocytomas, or myeloid leukemia cells (57).

The association of Hsp47 with collagen production was underscored with the establishment of Hsp47 knockout mice (33). These mice were shown to be severely deficient in the mature, propeptide-processed form of alpha1(I) collagen and fibril structures in mesenchymal tissues. The molecular form of type IV collagen was also affected, and basement membranes were discontinuously disrupted in the homozygotes. The homozygous mice did not survive beyond 11.5 days postcoitus, and displayed abnormally orientated epithelial tissues and ruptured blood vessels. When triple helix formation of type I collagen secreted from cultured cells was monitored by protease digestion, the collagens of $Hsp47^{+/+}$ and $Hsp47^{+/-}$ cells were resistant to protease digestion, but those of Hsp47-/- cells were sensitive. These results indicated for the first time that type I collagen is unable to form a rigid triple-helical structure without the assistance of the molecular chaperone Hsp47, and that mice require Hsp47 for normal development (33).

## 6. COLLAGEN BINDING PROPERTIES OF HSP47

Hsp47 was first identified by its ability to bind to gelatin, and was later shown to bind to triple helical collagen I (10). The affinity of Hsp47 for native collagen types-I, -II, -III -IV, and -V (I-V) has been studied using a BIAcore™ system that allows quantification of protein-protein interactions. Intriguingly, collagens I-V were all shown to have a similar affinity for Hsp47, with dissociation constants of $\sim 10^{-7}$ M. The low dissociation constant results from rapid dissociation and a relatively high association rate constant. These features seem to stipulate high cellular concentrations of Hsp47 for dedicated Hsp47-collagen interactions (58) .

The protein crystal structure of Hsp47 has yet to be completely resolved, however, based on the structure of protein C inhibitor, which shares 31% sequence identity and 70% similarity, Hsp47 is predicted to possess a long cleft that could accommodate most collagen chains (44). Binding of Hsp47 to gelatin and native collagen is pH-contingent, and is abolished below pH 6.3 (59). In fact, Hsp47 undergoes reversible pH-induced conformational changes that can be measured by circular dichroism (60). Post-translational modification of Hsp47 by glycosylation

or phosphorylation is not required for collagen binding in that recombinant Hsp47 produced in prokaryotes have similar binding properties to native Hsp47 (58, 61).

The location(s) of Hsp47 binding sites on the collagen molecule are, as yet, incompletely defined. Hsp47 has been shown to be partially eluted from gelatin- and native-collagen sepharose with an RGDS peptide suggesting that Hsp47 recognizes the integrin-binding RGD sequence. However, other determinants also appear to be involved with collagen triple helix –Hsp47 binding as high concentrations of RGDS peptides only displace ~50% of the bound Hsp47 (62). The intact alpha1(I) N-propeptide isolated from bone is also a competitive inhibitor of Hsp47 gelatin binding. Although a substantial proportion of alpha 1(I) N-propeptide/Hsp47 binding was attributed to the triple helical sequence of this domain, the N-terminal globular region of the N-propeptide (aa 23-108) also had Hsp47 binding properties (63).

Most lately, the substrate specificity of Hsp47 was examined *in vitro* using well-characterized CNBr peptide fragments of type I and type II collagen along with radiolabeled, recombinant Hsp47. Interaction of these peptides with Hsp47 bound to collagen-coated microtiter wells showed several binding sites for Hsp47 along the length of the alpha1 and alpha2 chains of type I collagen and the alpha1 chain of type II collagen, with the N-terminal regions showing the strongest affinities. The latter observation was also supported by the results of a ligand-blot assay. Except for two peptides in the alpha2(I) chain, peptides that showed substantial binding to Hsp47 did so in their triple-helical and not random-coil form. Thus, suggesting that additional structural requirements exist for Hsp47 binding besides the known preference for third-position Arg residues and the triple-helical conformation (64, 65).

## 7. HSP47 IN THE BIOSYNTHESIS OF COLLAGEN

The earliest association of Hsp47 with collagen appears to be co-translational and occurs during import into the ER, in that immunoprecipitation with anti-Hsp47 antibodies results in co-immunoprecipitates of Hsp47 with nascent proalpha1(I) chains still associated with polysomes (49, 63). These studies indicated that Hsp47 may be necessary to maintain the emerging peptides in an unfolded state until synthesis is complete, and then contribute to the formation of large transient aggregates containing newly synthesized procollagen (66). This potential role in protein translocation is analogous to that described for BiP, which seems to involve multiple BiP/substrate binding as well as release measures and BiP transactions with Sec63, a membrane component of the translocation machinery (67). Interestingly, Sec61 alpha, a multispanning membrane translocon protein that has been implicated as essential for translocation of polypeptides chains into the cisterns of the ER, has been recovered in a protein complex with collagen and Hsp47 in F9 cells treated with retinoic acid for 72 hours (68, 69). However, additional functional studies will be essential to substantiate a role of Hsp47 in collagen translocation.

Recently, it was shown that Hsp47 is able to bind to a monomeric prolyl peptide inducing the formation of a polyproline type II conformation. In doing so, it has been proposed that Hsp47 is able to induce the formation of higher order assemblies of the peptide with increased stabilities (70). Translation of these activities into biological function suggest that Hsp47 may bind to nascent monomeric polypeptide chains, inducing a productive polyproline type II conformation that aids the formation of the triple helix. Equally, Hsp47 may bind to misfolded areas within fully formed collagen that are proposed to be the result of collagen folding, reducing possible intramolecular aggregation, while again inducing polyproline type II conformation and assembly (70). Which combination of these functions Hsp47 provides within the cell has yet to be completely discerned.

Hsp47 continues to bind to procollagen chains retained in the ER when folding of the triple helix is prevented by incubation with the proly 4-hydroxylase inhibitor, alpha, alpha'-dipyridyl (59). However, this does not signify that Hsp47 participates in surveillance for altered collagens, in that Hsp47 binds equally well with unfolded and helical collagen (40). More than likely, other molecular chaperones such as BiP (71) and GPR94 that stably associate with unfolded and misfolded proteins, including collagen (49), play a monitoring role leading to ER retention of collagen (72).

The intracellular site of Hsp47 release from procollagen has been elegantly delineated using vesicular transport inhibitors to block the secretory pathway (59, 73). These studies revealed that procollagen retained in the ER by treatment with brefeldin A, calphostin C, or mastoparan, continues to bind Hsp47. Hsp47 also remains bound to procollagen within pre-Golgi intermediate vesicles in cells treated with guanosine 5'-3O-(thio)triphosphate, which blocks uncoating of both ER- and Golgi-derived transport vesicles. However, no Hsp47 binding is detected when transport between the medial-Golgi and trans-Golgi is blocked by monensin (40). Thus, two plausible mechanisms of release are advocated by these compartmental binding characteristics. One possibility is that Hsp47 is localized in the ER, and the rapid dissociation rate constant foresees that in the absence of free Hsp47, procollagen bound-Hsp47 would be rapidly released in the *cis*-Golgi. Another alternative is that since Hsp47/collagen binding *in vitro* is disrupted below pH 6.3, any remaining Hsp47 bound to collagen would dissociate in the Golgi where the pH has been estimated to be approximately 5.7 (74).

There is hard evidence that within the Golgi, procollagen molecules begin to align themselves to form large dense aggregates. Moreover, these aggregates are seen in all compartments of the Golgi and have been shown to correlate with the release from Hsp47, suggesting that, as its levels diminish Hsp47 may exhibit anti-charperone characteristics and aid in further aggregate formation (74). Collagen aggregates are also seen on the surface during secretion, which is thought to facilitate fibril formation (74). Although procollagen can be secreted in the absence

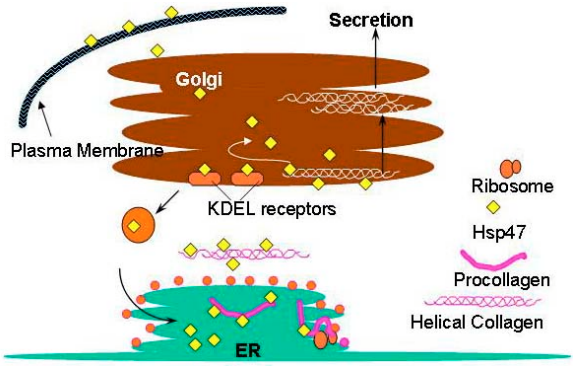


**Figure 1.** Schematic representation of the possible functions of Hsp47. Hsp47 is closely located with the translation and translocation of procollagen molecules. The continual association in the ER and cis-Golgi prevents aggregation and facilitates modification of collagen molecules. The persistent binding of Hsp47 to collagen prevents degradation of molecules prior to fibril formation. Dissociation of Hsp47 from collagen occurs beyond the cis-Golgi, where upon Hsp47 is recycled back to the ER. In epithelial tissues, some forms of malignancy and autoimmune connective tissue diseases Hsp47 is not recycled to the ER but is expressed on the cell surface.

of Hsp47 (75), it is not known whether this impedes the ability of these molecules to form fibrils in the extracellular spaces. It should be noted that most of our perspectives on the role of Hsp47 in collagen formation have been based on studies and observations involving type I and type III collagen, which form fibrils. The demonstration of Hsp47 at the cell surface in cells producing basement membranes collagen, such as collagen type IV, as originally described by Kurkinen *et al* (10) has yet to be adequately addressed. **Figure 1** depicts a summary of Hsp47's role in collagen biosynthesis.

## 8. HSP47 IN DISEASE

### 8.1. Fibrotic Diseases

Extensive depositions of collagen(s) are a hallmark feature of a broad group of diseases affecting many different organs and organ systems. To study the process of fibrosis a number of animal models have been developed and most of these have been distinguished by increased levels of Hsp47 expression. For example, the use of anti-Thy-1 antibody injections in rats produces early mesangiolytic changes followed by diffuse glomerulosclerosis in the kidney. These changes have been shown to be the result of an increased deposition of collagen type IV with increased expression of Hsp47 in the proliferative and sclerotic glomeruli (76). Similar relationships have been shown to occur in hypertensive nephropathy and the remnant kidney model of renal fibrosis where up-regulation of Hsp47 is associated with increased collagen biosynthesis in most intra-glomerular cells of the remnant kidney (77, 78). Similar patterns of Hsp47 and collagen up-regulation have been demonstrated in the development of age-related nephropathy, lipid nephropathy, cisplatin nephropathy, gentamicin nephropathy and unilateral ureteral obstruction (79-85). In addition, Hsp47 has been shown to be up-regulated and co-localized in cells producing excessive levels of collagen type III in various pulmonary fibrosis models (86). Also, both type-I and type-III collagens have been associated with increased Hsp47 in Itoh cells in livers undergoing fibrosis or cirrhosis (87, 88).

Cicatricial pemphigoid (CP) is an autoimmune mucocutaneous blistering disease associated with scarring. In one limited study, the expression of TGF-beta 1, Hsp47, type-I collagen and type-III collagen have been shown to be up-regulated in the fibrotic skin of CP patients (89-91). Likewise, epidermolysis bullosa acquisita (EBA) is a chronic sub-epidermal blistering disease involving the skin and mucous membranes that heal with scar formation and milia. Razzaque et al. (92) have recently shown on up-regulation in the production of collagens type-I and -III, Hsp47 , MMP-1, MMP-14, and TIMP-1, 2 and 3 during the process of conjunctival matrix remodeling in patients with EBA.

Also, the expression levels of collagen type-I and -III, and Hsp47 has been examined in keloid lesions and surrounding unaffected skin. Collagen type-I and -III mRNA levels were found to be up-regulated 20-fold in keloid tissues, contradicting previous reports of nearly normal type III collagen levels in this disease. Hsp47 expression in keloid lesions was also highly up-regulated; eightfold at mRNA level and more than 16-fold at the protein level. Strong up-regulation of these three proteins in keloid was confirmed by immunohistochemical staining suggesting that accumulation of both type-I and type-III collagen is important for the development of keloid lesions, and that Hsp47 plays a role in the rapid and extensive synthesis of collagen in keloid tissues (93, 94).

### 8.2. Myocardial Injury and Vascular Disease

It is well accepted that geometric changes occur in the left ventricle after myocardial infraction influencing the function of the ventricle and the patient's prognosis (48). Three independent factors influence the development of the ventricular remodeling: infarct size, ventricular wall stress, and infarct healing (48). Animal studies have shown that, when myocardial infarction was produced in rats by ligation of left coronary artery, Hsp47 increased expression paralleled those of collagen type I and type III. More specifically, the expression of Hsp47 increased on day 2, reaching a maximum level around day 14 (3.5-fold higher compared with the preligation hearts), which was maintained at a high level up to day 28. Furthermore, *in-situ* hybridization showed Hsp47 expression in spindle-shaped mesenchymal cells located between surviving myocytes in the infarct's peripheral zone 24 h after the ligation, and in the entire infarct zone on day 14. These results were further substantiated by immunofluorescent staining. Interestingly, the expression of Hsp47 in cultured cardiac fibroblasts in hypoxic cultures was shown to be greater than that in normoxic cultures, indicating that hypoxia in myocytes is one of the factors which induces expression of Hsp47 (48) .

Strong focal expression of Hsp47 has been shown to be evident in atherosclerotic arteries, but not in normal arteries (95). However, a proportion of Hsp47-expressing cells in atherosclerotic plaques were shown not to express type-I procollagen. This unique pattern of Hsp47 expression could be reproduced in culture following heat shock or treatment with oxidized low density lipoproteins, both of which promoted the expression of Hsp47 by smooth muscle cells. Importantly, these increases occurred without a concurrent rise in proalpha1(I) collagen expression. Localization of Hsp47 to the fibrous cap, its regulation by growth factors in parallel with type I procollagen, and its selective up-regulation by stress suggested a role for Hsp47 as a determinant of plaque stability (96).

In assessing acute vascular restructuring Hsp47 expression was shown to be substantially up-regulated in carotid arteries injured by balloon catheterization, with intense immunostaining in neointimal smooth muscle cells (SMCs). Hsp47 expression in SMCs was also correlated with the emergence of a less mature phenotype and with expression of type I procollagen. Interestingly, a sharp decline in Hsp47 expression was evident after carotid artery injury, with the appearance of collagen fibrils in the local extracellular matrix. Furthermore, type-I collagen fibrils, but not collagen monomers, inhibited expression of Hsp47 by SMCs. These findings indicate that up-regulation of Hsp47 is a feature of vascular restructuring, including acute neointimal formation, and that the constituents of the extracellular matrix regulate the duration of expression.

Recently, Rocnik *et al.* (97) demonstrated that over-expressing Hsp47 in vascular SMCs results in type-I procollagen being secreted faster than SMCs transduced with empty vector, yielding a greater accumulation of pro alpha1(I) collagen in the extracellular space. Interestingly, the amount of intracellular pro alpha1(I) collagen was also increased. This was associated with an unexpected increase in the rate of pro alpha1(I) collagen chain synthesis and 2.5-fold increase in pro alpha1(I) collagen mRNA expression. This amplification of procollagen expression, synthesis, and secretion by Hsp47 gave SMCs an enhanced capacity to elaborate a fibrillar collagen network. These effects of Hsp47 were qualitatively distinct from, and independent of, those of ascorbate and the combination of both factors yielded an even more intricate fibril network. Most notable was that when evidence for inter-individual variability in Hsp47 expression was sought, a common single nucleotide polymorphism ((−656)T) within a retinoic acid-responsive element in the Hsp47 gene promoter was discovered among African Americans that significantly reduced promoter activity. In a larger sample of African patients (n = 162), the frequency of the (−656)T allele was 0.11 (97).

### 8.3. Rheumatoid Diseases

Levels of Hsp47 protein and autoantibodies to Hsp47 in the sera of patients with rheumatic autoimmune diseases, including rheumatoid arthritis, systemic lupus erythematosus, Sjogren's syndrome, and mixed connective tissue disease (MCTD), have been assessed by enzyme-linked immunosorbent assay and immunoblot analysis. In these studies, Hsp47 antigen and autoantibody levels were significantly elevated in the sera of the rheumatic autoimmune disease patients, but not in the sera of the idiopathic pulmonary fibrosis patients. The sera of the MCTD patients showed particularly high levels of Hsp47 antigen relative to healthy controls (1.99+/-0.22 vs 0.41+/-0.07 ng/ml). Autoantibodies to Hsp47 were also in high levels in the sera of MCTD patients, suggesting that simultaneous occurrence of systemic inflammation and up-regulation of Hsp47 caused leakage of Hsp47 from fibrotic lesions into the peripheral blood, and the leaked antigen induced high titer of autoantibodies to Hsp47. These findings intimate that levels of HSP47 antigen and autoantibody may prove to be useful adjunct biomarkers of MCTD (98). Notable, is that two types of 47 kDa antigen are specifically recognized in sera from rheumatoid arthritis (RA) patients (99). An N-terminal amino acid sequence in one of the 47 kDa antigens, RA-A47, possessed 81% homology to that deduced from the DNA sequence of the colligin gene which is reported as human Hsp47 gene (CBP2), and 100% homology to that deduced from the DNA sequence of colligin-2 gene, a homologue of colligin. The RA-A47 cross-reacted with a monoclonal antibody raised against chick Hsp47 and bound to gelatin (100). The expression of the RA-A47 gene was enhanced by heat shock treatment and TGF-beta stimulation, suggesting that RA-A47 is a Hsp47-like protein, presumably the product of the colligin-2 gene. It was hypothesized that a collagen-specific molecular chaperone(s) such as Hsp47 and/or RA-A47 is involved in cartilage destruction in RA (101). Subsequently, it has been shown that RA-A47 protein is specifically down regulated in RA, causing the intracellular accumulation of unsecretable type-II collagen, while the extracellular matrix (ECM) is degraded by MMPs and iNOS through the stimulation of chondrocytes by TNFalpha. The altered localization of RA-A47 to the surface or outside of cells is believed to represent the mechanism for the recognition of RA-A47 as an autoantigen during rheumatoid arthritis (102).

### 8.4. HSP47 and Cancer

Hsp47 was shown early on to be down-regulated along with collagen synthesis when fibroblasts were transformed with Rous Sarcoma virus, simian virus 40, or c-Ha-ras oncogene (43). Conversely, teratocarcinoma cells forced to differentiate with retinoic acid and/or dibuteryl cyclic AMP result in a greater production of collagen IV and a concurrent up-regulation of Hsp47 (52). Later Morino et al. (103-109) showed that Hsp47 was associated with solid sarcomas rather than tumor ascites. This is not surprising in that sarcomas produce collagen as an integral part of the neoplasm. Moreover, when the expression of Hsp47 was related to tumor aggressiveness, less aggressive tumors expressed more Hsp47 than more indolent lesions; however, Hsp47 was also expressed in metastases as well as primary lesions (109).

Hsp47 has also been observed among osteosarcomas(110). Overall survival was compared between groups over-expressing or not HSPs using

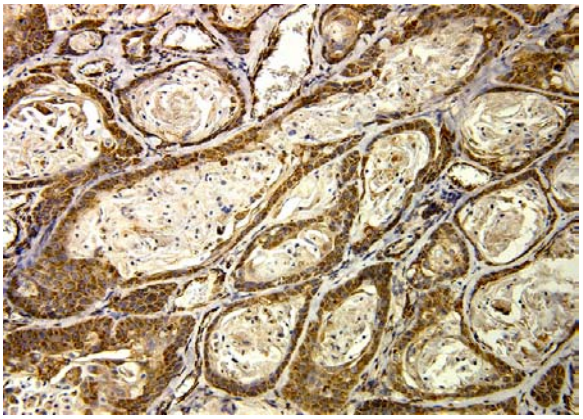

**Figure 2.** Well-differentiated squamous cell carcinoma stained with anti-Hsp47 antibodies. Staining is limited to the advancing edge of the tumor islands and of some stromal elements. No staining is observed in the central well-differentiated keratinocytes. Original Mag. 20X.

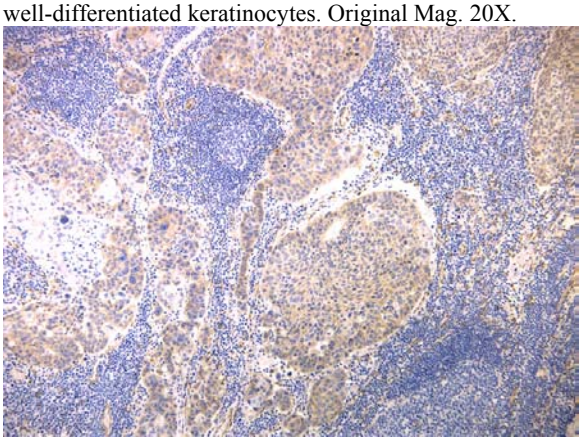

**Figure 3.** Lymph node containing moderately differentiated squamous cell carcinoma stained with anti-Hsp47 antibodies. Tumor cells are diffusely stained throughout the lymph node. Lymphocytes exhibit no stained for Hsp47. Orignal Mag. 20X.

Wilcoxon's test and Cox's proportional hazard model. The over-expression rate at biopsy was 22% (Hsp27), 88% (Hsp47), 66% (Hsp60), 48% (Hsp70), 47% (Hsp90alpha), 31% (Hsp90beta), and 17% (p53), respectively. The rate at surgery was 33% (Hsp27), 94% (Hsp47), 60% (Hsp60), 49% (Hsp70), 28% (Hsp90alpha), 40% (Hsp90beta), and 17% (p53), respectively. However, only Hsp27 and p53 over-expression at biopsy had a negative prognostic value. Hsp27 showed the strongest negative prognostic value in osteosarcoma.

Most recently, an extended analysis of the results of a previous microarray analysis by immunohistochemical validation of differential protein expression in a series of 57 surgically resected infiltrating ductal pancreatic adenocarcinomas was performed (111). Two representative genes were examined: sea urchin fascin homolog (over-expressed in both cell lines and primary tumors) and Hsp47 (over-expressed in primary tumors only). Protein expression also was evaluated in the precursor lesions of pancreatic cancer ( i.e. pancreatic intraepithelial neoplasia (PanIN)), and normal ductal epithelium. Fascin expression was seen in the neoplastic cells of 54 (95%) of 57 ductal adenocarcinomas but not in 49 (94%) of 52 adjacent nonneoplastic epithelium. In the multistep pathogenesis of ductal adenocarcinomas, fascin expression seemed to be a late event, usually present in PanINs 2 and 3. Hsp47 expression was almost universal and most intense in the ductal adenocarcinoma-associated stromal desmoplasia (57/57), although 37 cases (65%) also expressed Hsp47 in the neoplastic epithelium. Hsp47 expression was absent in the majority of nonneoplastic pancreata (46 (88%)). This is the first study that demonstrated that Hsp47 along with Fascin are novel tumor markers with potential diagnostic and therapeutic implications for pancreatic carcinoma (111).

Subsequent to recognition that Hsp47 (CBP2) mapped to human chromosome 11q13.5 , a locus frequently over-expressed in squamous cell carcinomas of the head and neck, Hsp47 was shown to be commonly expressed in head and neck carcinomas (112). Hsp47 has been localized to the cell surface in many head and neck cancer cell lines, where it is anchored in a complex with CD9 (113). However, the functional significance of its cell surface expression is yet unclear. Among solid tumors, squamous cell carcinomas (SCCs) displayed a characteristic pattern of staining with Hsp47 localized along the advancing edge but not within the central differentiated regions of the tumor cell islands (Figure 2) (114). However, diffuse staining of Hsp47 was observed in tumors that lacked well-formed tumor islands. Noteworthy, is that all positive stained metastatic lymph nodes lacked well-formed islands and showed a diffuse pattern of immunoreactivity (Figure 3). Similar to the desmoplastic stroma of pancreatic duct adenocarcinoma, the oral SCCs demonstrated varying degrees of Hsp47 staining in stromal cells and adjacent fibroblasts (114). More recently, we have observed Hsp47 expression among both benign and malignant salivary gland neoplasms (Figures 4).

Treatment of SCC cells with Hsp47 antisense phosphorothioate oligonucleotides has been shown to modulate the production of collagen XVIII and its carboxy terminus endostatin fragments, but not its expression, implying that Hsp47 may play a role in tumor progression by mediating the endogenous processing of collagen XVIII to a carboxy terminus 20 Kd fragment, endostatin, in tumor cells (55, 115). Consistent with this hypothesis, comparison between primary tumors and positive nodes of the metastatic cases revealed decreased expression of collagen XVIII and Hsp47 in metastases (114).

## 9. HSP47 AS A THERAPEUTIC TARGET

Based on *in vitro* studies demonstrating that antisense inhibition of Hsp47 reduced collagen production and that Hsp47 knockout mice produced diminished and inadequate collagen, a number of clinical strategies have evolved targeting Hsp47 as a plausible means by which to limit fibrotic diseases. For example, treatment with

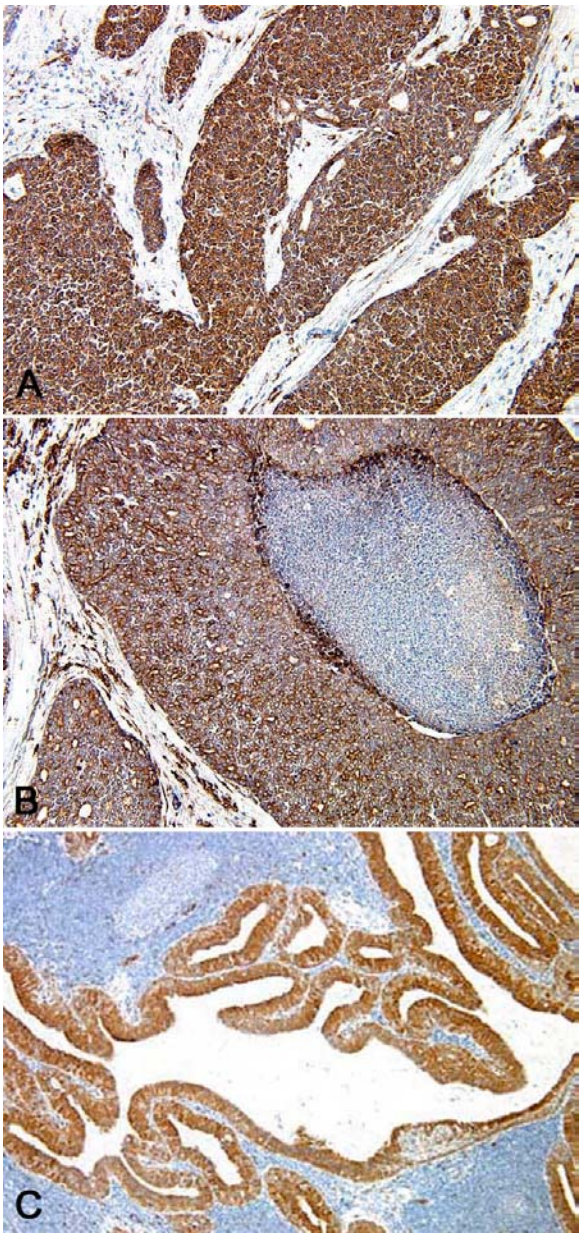


**Figure 4.** A. Solid form of adenoid cystic carcinoma from parotid salivary gland stained with anti-Hsp47 antibodies. Tumor islands depict staining throughout the cytoplasm of the tumor islands as well as stromal elements. B. Salivary duct carcinoma stained with anti-Hsp47 antibodies exhibiting staining of tumor cells and some stromal elements. C. Benign Warthin's tumor of parotid gland stained with anti-Hsp47 antibodies. The tumor cells, papillary cyst adenomas, show diffuse staining while the lymphocytic component of the tumor is unstained. Orignal Mag. 20X.

antisense oligonucleotides to Hsp47 abrogated chlorhexidine gluconate-induced changes in the expression of Hsp47, type-I and -III collagen, alpha-SMA, and in the number of infiltrating macrophages and vessels, and suppressed peritoneal fibrosis in a rat model system (116). Similar approaches have also been shown to be useful in controlling scar formation (117), glomerulosclerosis (118), pulmonary fibrosis, and cirrhosis (119) .

The identification of Hsp47 on the cell surface of many squamous carcinomas has suggested that this aberrant location of Hsp47 may have utility as a target for delivering chemotherapeutic agents or for gene vectors. Recently, it was demonstrated that doxorubicin (DOX) immunoconjugates that linked monoclonal antibodies (MAbs) against Hsp47 to the 13-keto position of the drug possessed high cytotoxic drug activity and antibody-directed killing of antigen bearing tumor target cells. The demonstration that SPA470-DOX is effective during hypoxia or conditions that mimic hypoxia presumes the further utility of anti-Hsp47-DOX in treating head and neck cancers (120).

Most recently, a novel type of ribozyme was developed by ligating a hammerhead sequence to a tRNA(Val) promoter to facilitate displacing the ribozyme from nucleus to cytoplasm and to a constitutive transport element, a binding motif of helicase, which unwinds mRNA to render the target sequence on the mRNA accessible to the ribozyme. This ribozyme showed strong activity to cleave Hsp47 mRNA and suppress the secretion of type I procollagen in the human primary fibroblast. However, this modality has yet to be tested in any disease model (121). However, it should be kept in mind that all strategies of Hsp47 targeting would seem to require tissue or organ targeting to have any utility, since system-wide effects of inhibiting Hsp47 may produce deleterious affects in many unaffected organs and tissues.

## 10. PERSPECTIVE.

The studies performed in Hsp47 knock-out mice have clearly demonstrated that Hsp47 is critical both for the production and maturation of collagens. In so doing, Hsp47 has been restricted to a very narrowly defined region, between the ER and *cis*-Golgi, of the cellular machinery responsible for collagen production and has been profoundly implied in fibril formation. However, little is known regarding the role of this novel serpin family member in the production and organization of non-fibrilar basement membrane collagens or for its relocation from an ER resident to a cell surface protein. In spite of these gaps, Hsp47 is evolving as a potential biomarker, target for various therapies including those controlling fibrotic diseases and, as a component for various drug delivery systems.

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