

## Research Paper

# Hsp60 expression, new locations, functions and perspectives for cancer diagnosis and therapy

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**Key words:** Hsp60 (Cpn60), tumor-cell survival, apoptosis, tumor diagnosis, differential diagnosis, assessing prognosis, monitoring response to treatment, chaperonotherapy, anti-tumor immune response, chaperonin, protein folding

Hsp60 in eukaryotes is considered typically a mitochondrial chaperone (also called Cpn60) but in the last few years it has become clear that it also occurs in the cytosol, the cell surface, the extracellular space and in the peripheral blood. Studies with prokaryotic models have shown that Hsp60 plays a role in assisting nascent polypeptides to reach a native conformation, and that it interacts with Hsp10 (which also resides in the mitochondria and is also named Cpn10). In addition to its role in polypeptide folding in association with Hsp10, other functions and interacting molecules have been identified for Hsp60 in the last several years. Some of these newly identified functions are associated with carcinogenesis, specifically with tumor cell survival and proliferation. Thus, assessing the levels of Hsp60 in tumor cells and in sera of cancer patients is becoming an attractive area of investigation aiming at the development of means for practical applications in clinical oncology. Since Hsp60 participates in extracellular molecular interactions and cell signalling and also in key intracellular pathways of some types of tumor cells, the idea of using Hsp60 in anti-cancer therapy (chaperonotherapy) is being investigated. The Hsp could be used either as an anticancer agent alone or in combination with tumor antigens, or as target for anti-chaperone compounds. In this article, a brief review is presented of representative research efforts aimed at assessing Hsp60 in a variety of tumors with the purpose of illustrating possible implications and applications for making early and differential diagnoses, assessing prognosis, monitoring response to treatment, and for developing novel anti-cancer strategies.

## Introduction

Heat shock proteins (Hsp) constitute a heterogeneous group of molecules with a variety of functions, many of which pertain to the response to stress and to protein folding. Historically, Hsps were considered stress proteins, with a subset of which being typically involved in assisting the correct folding of nascent polypeptides and in the refolding of partially denatured proteins resulting from cellular

stress.<sup>1-3</sup> These Hsps are the so called molecular chaperones. Very often the names Hsp and molecular chaperone are used interchangeably, although there are Hsps that have no known chaperoning function and, conversely, there are chaperones that do not belong to the Hsp group (these are proteins from genes that are not stress genes, i.e., they are not inducible by heat or any other known stressor).

Lately, the range of functions attributed to Hsp has greatly expanded to encompass participation in protein translocation, protein degradation, dissolution of pathologic protein aggregates, regulation of gene expression, cell differentiation, DNA replication, signal transduction, programmed cell death, cellular senescence and carcinogenesis. In addition, the concept of chaperonopathy was developed to indicate pathologic conditions due to quantitatively or qualitatively defective chaperones.<sup>4,5</sup> Furthermore, the use of chaperones in therapeutics, i.e., chaperonotherapy, for a variety of diseases is being investigated in many laboratories.<sup>6,7</sup>

A field currently very active is the study of the role of Hsps in carcinogenesis, and of the possible applications of Hsp detection and quantification in tissues and biological fluids for cancer diagnosis and, by extension, for assessing prognosis and monitoring disease progression and for developing antitumor therapeutic tools based on Hsps.<sup>8-10</sup>

Hsp60 is a molecular chaperone known to assist protein folding in prokaryotes and in eukaryotic cell organelles. The Hsp60 in the latter organelles is evolutionarily related to that present in bacteria, and is considered to have originated in the ancestors of some of today's extant species. Its role and applications in human cancer development and management are currently actively investigated and the results are very encouraging. Hsp60 seems to have potential in the areas of diagnosis-prognosis, and prevention and treatment of various human cancers. A brief review of representative findings made in the last several years is presented here to inform pathologists and clinicians and to stimulate basic and clinical research pertaining to Hsp60 and cancer. The data available suggest that Hsp60-based methods and reagents have potential to become valuable tools for practical application in oncopathology.

## Intracellular Hsp60

In eukaryotes, Hsp60 typically resides in the organelles mitochondria (chaperonin 60 or Cpn60) and chloroplasts (in plants),

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Submitted: 03/11/08; Revised: 05/12/08; Accepted: 05/13/08

Previously published online as a *Cancer Biology & Therapy* E-publication: <http://www.landesbioscience.com/journals/cbt/article/6281>

and interacts with Hsp10 (or Cpn10) while chaperoning nascent polypeptides as they progress to achieve a functional conformation or native status.<sup>11-13</sup> Hsp60 also interacts with mitochondrial Hsp70 (mortalin),<sup>14-16</sup> and with survivin and p53 as it participates in the process of apoptosis.<sup>17</sup> The levels of mitochondrial Hsp60 are regulated via complex mechanisms, one of which involves a DNA-dependent protein kinase, a molecule that plays a protective role against drug-induced apoptosis.<sup>18</sup>

In addition to its recognized role in protein folding, Hsp60 has been implicated in intracellular protein trafficking,<sup>16,19</sup> and in peptide-hormone signalling in an experimental model of Lyme neuropathy.<sup>20</sup> Hsp60 can bind and interfere with the functions of various endogenous and exogenous proteins. For instance, Hsp60 binds epolactaene, a microbial metabolite that if not bound to the chaperone arrests cell cycle and induces the outgrowth of neurites in human neuroblastoma cells.<sup>21</sup>

For many years Hsp60 was considered a typical intraorganellar chaperone. However, lately evidence has accumulated to show that Hsp60 is also found in the cytosol not only after mitochondrial release but also independently of such release, and the evidence also indicate that both, the mitochondrial and the cytosolic forms of Hsp60 can function in pro-survival or pro-apoptotic pathways, depending on the cellular situation.<sup>22</sup>

## Surface Hsp60

Hsp60 can also be found on the surface of normal<sup>23</sup> and tumor cells.<sup>24-26</sup> Increased amounts of Hsp60 on the cell's surface was considered to serve as a danger signal for the immune system leading to the activation and maturation of dendritic cells and the generation of an antitumor T-cell response.<sup>25,27</sup>

Quite interestingly from the oncological viewpoint, surface Hsp60 has been found associated with alpha-3-beta-1 integrin, a protein involved in the adhesion of metastatic breast cancer cells to lymph nodes and osteoblasts, with the association being inhibited by mizoribine, an Hsp60-binding drug.<sup>28</sup> It has also been shown that surface Hsp60 plays a critical part in the metastatization of pancreatic carcinoma,<sup>24</sup> and that presence of Hsp60 on the surface of oral tumor cells determines cell lysis induced by gamma-delta T lymphocytes.<sup>29</sup>

## Extracellular Hsp60

In addition to its intracellular and pericellular locations, Hsp60 also occurs in the extracellular space and in circulation, a fact that has become firmly established only in the last few years, although the mechanisms involved in its secretion, exosomal and/or other are not yet understood.<sup>30,31</sup>

Extracellular Hsp60 can interact with a number of cell-surface receptors such as CD14, CD40 and Toll-like-receptors (TLRs),<sup>32</sup> can cause pro- and anti-inflammatory effects,<sup>33,34</sup> and can bind to peptides (e.g., tumor-derived peptide antigens) and present them to immune system cells.<sup>35</sup> When acting as a pro-inflammatory agent, Hsp60 induces secretion of cytokines from professional antigen-presenting cells and causes activation of T cells.<sup>27,36</sup> Hsp60 can also stimulate the maturation of dendritic cells via the TLR-2 and -4 signal-transduction pathways,<sup>35</sup> synergizing the pro-inflammatory action of IFN $\gamma$ .<sup>37</sup>

Other reported effects of extracellular Hsp60 are induction of the release of TNF $\alpha$ , the production of nitric oxide, and the

induction of expression of the Th1-promoting cytokines IL-12 and IL-15 in macrophages.<sup>37</sup> However, it has to be noticed that sometimes pro-immune effects in experimental models might result from *Chlamydial*-Hsp60 tissue contamination.<sup>38</sup>

On the other hand, as anti-inflammatory agent, extracellular Hsp60 can be a ligand for gamma-delta T lymphocytes in oral tumors, determining antitumoral immunosuppression.<sup>39,40</sup> After stimulation by Hsp60, gamma-delta T cells showed downregulation of Fas expression and nitric oxide production, leading to a loss of mitochondrial membrane potential and caspase-9 activation, followed by induction of lymphocyte-cell death.<sup>40</sup>

Extracellular Hsp60 can also enter into the blood stream and be found in plasma of healthy subjects. In one study on 860 healthy members of the British Civil Service (457 men and 304 women), Hsp60 plasma levels, assessed by ELISA, were below 1 ng/mL in 46.9% of the subjects tested, between 1 and 1,000 ng/mL in 26.5%, and above 1,000 ng/mL in 26.6%. Molecular analyses showed that the circulating Hsp60 was the full-length protein lacking the mitochondrial import peptide, which suggested that the circulating Hsp60 originated in the mitochondria of cells that were not identified.<sup>41</sup>

In another study, it was found that high Hsp60 blood titres are correlated with low socioeconomic status, social isolation and, in women, psychologic distress.<sup>42</sup> However, the significance of Hsp60 in circulation with regard to tumors was not investigated.

## Hsp60 and Tumor Growth

Hsp60 favours the survival of certain types of tumor cells, and in some cases it may even be essential for tumor-cell growth. The need for Hsp60 by tumor cells has been revealed by various types of data. For example, elevated levels of this protein in tumor cells have been linked to: (i) ability to survive apoptotic stimuli; (ii) loss of replicative senescence; (iii) uncontrolled proliferation; and (iv) neoplastic transformation.<sup>17,43-45</sup> In addition, it has been shown in *in vitro* experiments that Hsp60 knockdown arrests osteosarcoma cell growth.<sup>15</sup> Likewise, inhibition of Hsp60 expression by short hairpin RNA plasmids stopped tumor cell growth.<sup>14</sup>

Tumor immunogenicity may also depend on whether tumor cells express and secrete Hsp60 or not.<sup>46</sup> This observation alerts us to the potential predictive usefulness of monitoring levels of Hsp60 in cells and biological fluids in order to assess the probability that an effective immune response against a tumor will be elicited.

It is well established for some malignancies, both *in vitro* and *in vivo*, that Hsp60 is cytoprotective for tumor cells by increasing the apoptotic threshold.<sup>17</sup> It is also known that Hsp60 can enhance caspase activation<sup>47,48</sup> or conversely, stimulate anti-apoptotic mechanisms involving sequestration of Bax-containing complexes.<sup>49</sup> Thus, Hsp60 can have opposite effects with regard to tumor cell survival. Consequently, Hsp60 may be defined as "the molecular Proteus" of tumor cell survival.<sup>44</sup>

It has been shown in an *in vitro* model, that Hsp60 expression in breast cancer cells is decreased by collagen V, a component of normal human breast stroma that is over-deposited in ductal infiltrating carcinoma.<sup>50</sup> Interestingly, collagen V induced apoptosis of tumor cells, but the mechanism responsible for it and for the Hsp60 decrease has not yet been clarified. These are topics that deserve investigation to elucidate to what extent, if any, collagenogenesis affects tumor progression *in vivo* and whether a decrease in Hsp60

favors tumor cell death or the contrary in breast ductal infiltrating carcinoma.

## Hsp60 in Human Tumor Specimens: Diagnostic and Prognostic Implications

A variety of tumors from a range of tissues have been examined to look for the presence and levels of Hsp60, and the results indicate that detection and quantification of this Hsp can provide useful information for establishing histopathological diagnosis and assess prognosis. A compendium of results is displayed in Tables 1 and 2, and some are discussed below, tissue by tissue.

**Tumors of the nervous system.** Hsp60 was shown by RT-PCR to be overexpressed in astrogloma cells.<sup>51</sup> In contrast, in another study, using proteomics, it was found that the levels of Hsp60 are low in multiform glioblastomas as compared to the levels in non-tumor tissues.<sup>52</sup> It was proposed that a better characterization of Hsp60 involvement in tumor of the nervous system could be useful for differential diagnosis and for predicting response to therapy.

**Hematolymphopoietic tumors.** Hsp60 is widely distributed in hematolymphopoietic tissues. Its expression in bone marrow has been found to correlate with B-cell maturation,<sup>53</sup> but not with maturation of the myeloid and megakaryocytoid lineages.<sup>54</sup> Thus, Hsp60 determination in bone marrow would offer a means of distinguishing cell lineages in bone marrow specimens.

Hsp60 levels were investigated in acute lymphoblastic leukaemia but no noteworthy changes were observed.<sup>53</sup> By contrast, in blasts of acute myeloid leukaemia (AML), Hsp60 levels were found augmented and correlated with increased levels of CD14, CD15, CD33 and CD34, and associated with a poor prognosis in those patients carrying unfavorable karyotypes.<sup>55</sup>

Hsp60 was found increased in malignant circulating cells of patients with AML compared with those from chronic myeloid leukaemia (CML) patients and with normal peripheral-blood mononuclear cells.<sup>56</sup> Thus, assessing levels of Hsp60 in circulating blasts has potential as a means for distinguishing blasts of AML from blasts of CML.

Hsp60 was found, by immunohistochemical and Western blotting analyses, abundant in Hodgkin's lymphomas and scarce in small lymphocytic lymphomas, anaplastic large cell lymphomas and immunoblastic lymphomas.<sup>57</sup> These findings suggest that determination of Hsp60 levels could aid in the differential diagnosis of malignant lymphopathies.

**Airway tumors.** Hsp60, as demonstrated by immunohistochemistry and Western blotting, becomes undetectable during bronchial carcinogenesis in smokers with chronic obstructive pulmonary disease.<sup>58,59</sup> Hsp60 was present in normal and hyperplastic bronchial epithelium but, in comparison, its levels were low in squamous metaplasia, and it was undetectable in dysplasia and adenocarcinoma specimens.<sup>59</sup> Interestingly, Hsp60 was also undetectable in normal epithelium in proximity of cancerous tissue.<sup>58</sup> The data reported indicate that distinct Hsp60 levels associated with cell-tissue type could be distinctive markers of tissue-lesion type and stage in the respiratory tract.

**Oral tumors.** No differences in Hsp60 levels were found between normal odontogenic epithelium and ameloblastomas.<sup>60</sup> It would, therefore, appear that examining tissue levels of Hsp60 provides no help in distinguishing normal from malignant dental tissues.

Hsp60 has been found to be involved in inflammatory processes that take place during proliferation of the oral lining epithelium, leading to progression of periapical inflammatory lesions.<sup>61</sup> In contrast, Hsp60 expression was not found to be associated with carcinogenic steps in a retrospective immunohistochemical study of the dysplasia-carcinoma sequence of oral mucosa.<sup>62</sup> Therefore, measuring Hsp60 expression levels in oral tissues would have no diagnostic value with regard to oral cancer. The same conclusion was derived from measurements of Hsp60 levels by ELISA in patients' sera for diagnostic screening of pre-neoplastic oral lesions.<sup>63</sup>

A different outlook was provided by other studies. Hsp60 was assessed immunohistochemically in the basal and suprabasal epithelium of the oral mucosa and its levels were found significantly higher in oral liken planus than in oral fibromas.<sup>64</sup> Hsp60 was absent in squamous cell carcinomas of the tongue, while it was present in normal epithelium and present but discontinuously in dysplastic lesions, suggesting that production of the Hsp decreased (due to gene downregulation and/or other mechanisms accompanying tumorigenesis) progressively with the progression of carcinogenesis.<sup>65</sup> These data show that low Hsp60 ought to be considered a warning sign in evaluating tongue lesions since low levels of this protein could indicate that malignant transformation is in progress.

**Oesophageal tumors.** High levels of Hsp60 were observed in oesophageal squamous cell carcinomas and were considered to be indicators of good prognosis.<sup>66</sup> Hsp60 was found elevated in tumor tissue compared to normal mucosa, and the high levels of this Hsp correlated with an increment of the apoptotic index and with a better five-year survival rate.

**Gastric tumors.** Hsp60 is a major target antigen for the immune system in mucosa-associated lymphoid tissue (MALT) lymphomas.<sup>67</sup> In these lymphomas, Hsp60 has often been found, using immunohistochemistry, in follicular dendritic cells.<sup>68</sup> The Hsp60 of *Helicobacter pylori* (HP) is very similar to human Hsp60 and the immune response induced by these two crossreactive molecules has been implicated in the development of gastric MALT lymphoma (MALToma).<sup>67,69</sup> HP-Hsp60 induced IL-8 secretion in gastric epithelial cells.<sup>70</sup> Moreover, the levels of IgG anti-HP-Hsp60 were significantly elevated and correlated with IgG anti-HP levels in patients with MALTomas.<sup>71</sup> It has also been found that measuring the levels of pre-treatment serum IgG antibodies against HP-Hsp60 in patients with gastric MALToma helped in predicting the regression, or lack thereof, of the tumors after HP eradication.<sup>72</sup>

**Large bowel tumors.** Hsp60 is elevated during colorectal carcinogenesis and tumor progression, as shown by immunohistochemistry (Fig. 1A) and western blotting,<sup>73,74</sup> and by cDNA microarray analysis.<sup>75</sup> The data suggest that immunohistochemistry helps to distinguish normal from dysplastic glands in tubular adenomas,<sup>73</sup> while Western blotting can only distinguish colonic polyps from normal tissue.<sup>76</sup> Hsp60 levels have also been correlated with tumor grade and stage<sup>74,75</sup> and with occurrence of lymph node metastases (Fig. 1B).<sup>74</sup> The higher was the level of Hsp60 in tumor specimens, the more advanced were the grade and the stage of the tumors. Metastatic lymph nodes showed a higher percentage of cells positive for Hsp60 in the secondary follicles than the hyperplastic lymph nodes. A recent study showed that tissue Hsp60, detected by immunohistochemistry, and anti-Hsp60 antibody titres in serum measured by ELISA were significantly higher in patients with colorectal cancers compared to controls.<sup>77</sup>

**Table 1 Tumors in which the levels of Hsp60 have been studied providing information directly pertinent to the diagnosis**

System	Tumor	Methods <sup>a</sup>	Hsp60 levels	Ref.	
Nervous	Astrogloma	RT-PCR	Elevated	51	
	Glioblastoma	Proteomics	Decreased	52	
Haemolympho-poietic	Acute lymphoblastic leukaemia	2D-gel electrophoresis	No changes	53	
	Acute myeloid leukaemia	Flow cytometry	Elevated	55, 56	
	Chronic myeloid leukaemia	Flow cytometry	No changes	56	
	Hodgkin's lymphoma	IHC, WB	Elevated	57	
	Small lymphocytic lymphoma	IHC, WB	No changes	57	
	Anaplastic large cell lymphoma	IHC, WB	No changes	57	
	Immunoblastic lymphoma	IHC, WB	No changes	57	
Respiratory	Bronchial adenocarcinoma	IHC, WB	Decreased	58, 59	
Digestive	Ameloblastoma	IHC	No changes	60	
	Oral carcinoma	IHC, ELISA	No changes	62, 63	
	Oral liken planus	IHC	Elevated	64	
	Fibromas	IHC	No changes	64	
	Tongue carcinoma	IHC	Decreased	65	
	Oesophageal squamous carcinoma	IHC	Elevated	67	
	Gastric MALToma	IHC	Elevated <sup>b</sup>	68,69	
	Large bowel	IHC, WB, cDNA microarray, ELISA	Elevated	73-75, 77	
	HCV-hepatocellular carcinoma	Proteomics	Elevated	78	
	HBV-hepatocellular carcinoma	IHC, dot-immunoblot	No changes	79	
	Pancreatic ductal adenocarcinoma	Proteomics	No changes	80	
	Urinary	Vesical transitional cell carcinoma	IHC	Decreased	81,82
		Carcinosarcoma	IHC	Decreased	84
Male reproductive	Prostate adenocarcinoma	IHC	Elevated	85, 86	
Female reproductive	Exocervical carcinoma	IHC, WB	Elevated	89, 90	
	Ovarian carcinoma	IHC	Elevated	88,89	
Endocrine	Breast ductal invasive carcinoma	2D-gel electrophoresis	Elevated	94	
	Adrenal cushing tumor	IHC	Elevated	97	
Skeletal	Osteosarcoma	IHC, ELISA	Elevated or no changes	99-101	
	Condroma	IHC	No changes	102	
	Condrosarcoma	IHC	No changes	102	

<sup>a</sup>Abbreviations: RT-PCR, real time PCR; IHC, immunohistochemistry; WB, Western blotting; Ref., reference. <sup>b</sup>Both, human- and *Helicobacter pylori* Hsp60 are elevated.

**Tumors of the liver and pancreas.** Hsp60 levels were assessed by proteomics and found increased in specimens of hepatocellular carcinomas (HCCs) from patients with hepatitis C virus (HCV) infection, compared with non-cancerous HCV-infected liver tissue.<sup>78</sup> In contrast, Hsp60 levels determined by immunohistochemistry and dot immunoblotting were not increased in specimens of hepatitis B virus (HBV) infected patients with HCCs, compared with non-tumoral HBV-infected liver tissues.<sup>79</sup> These two reports show once again that the method used for assessing Hsp60 should be taken into consideration in evaluating the results of measuring the levels of this Hsp; one method can show increase while another may not. This kind of results also indicate that method selection could be critical in designing strategies for studying malignant pathological conditions in which Hsp60 levels might be altered.

Hsp60 has been found by proteomics to occur in normal exocrine parenchyma and ductal adenocarcinomas of pancreas but no correlation was observed between Hsp60 levels and tumor differentiation grade.<sup>80</sup>

**Urinary tract tumors.** Hsp60 has been found, by immunohistochemistry, in tumor specimens at low levels or absent in patients with vesical transitional-cell carcinomas.<sup>81,82</sup> Hsp60 levels were correlated with outcome, the lower Hsp60 levels the worse was the outcome after local treatment of superficial bladder carcinomas.<sup>81</sup> Another research group showed in invasive or high-risk superficial bladder cancers that high levels of Hsp60, as detected by immunohistochemistry prior to neoadjuvant chemoradiotherapy, predict a good response to treatment.<sup>83</sup> In vesical carcinosarcomas, reduced levels of Hsp60 were found by immunohistochemistry in comparison with those in normal bladder mucosa.<sup>84</sup> The data suggest that measuring Hsp60 levels have potential in the histopathologic identification of bladder carcinosarcomas particularly at early stages.

**Tumors of the male reproductive system.** Increased levels of Hsp60 is an early sign of prostatic carcinogenesis as demonstrated by immunohistochemistry in low- and high-grade prostatic intraepithelial lesions, as well as in prostatic adenocarcinomas (Fig. 1C).<sup>85</sup>

**Table 2 Examples of tumors in which the levels of Hsp60 have been studied providing information useful in predicting prognosis**

Tumor	Hsp60 levels	Correlation with patients' outcome	Ref. <sup>a</sup>
Acute myeloid leukaemia	Elevated	Worse prognosis in patients with unfavourable phenotype	55
Oral carcinoma	No changes	Scarce utility	63
Oesophageal squamous carcinoma	Elevated	Elevated correlates with a better five-year survival	64
Gastric MALToma	Elevated	<i>Helicobacter pylori</i> (HP) Hsp60 elevated predicts tumor regression after HP eradication	72
Vesical transitional cell carcinoma	Decreased	Lower levels correlate with worse outcome of local treatments	81
		Higher levels prior to neoadjuvant chemoradiotherapy correlate with better treatment response	83
Prostate adenocarcinoma	Elevated	No correlation between Hsp60 levels and outcome	87
Ovarian carcinoma	Elevated	Higher levels correlate with worse prognosis in patients treated with cisplatin-containing chemotherapy	92
		Higher levels correlate with earlier stage and better outcome	93
Osteosarcoma	Elevated (or not)	No correlation with outcome	100, 101

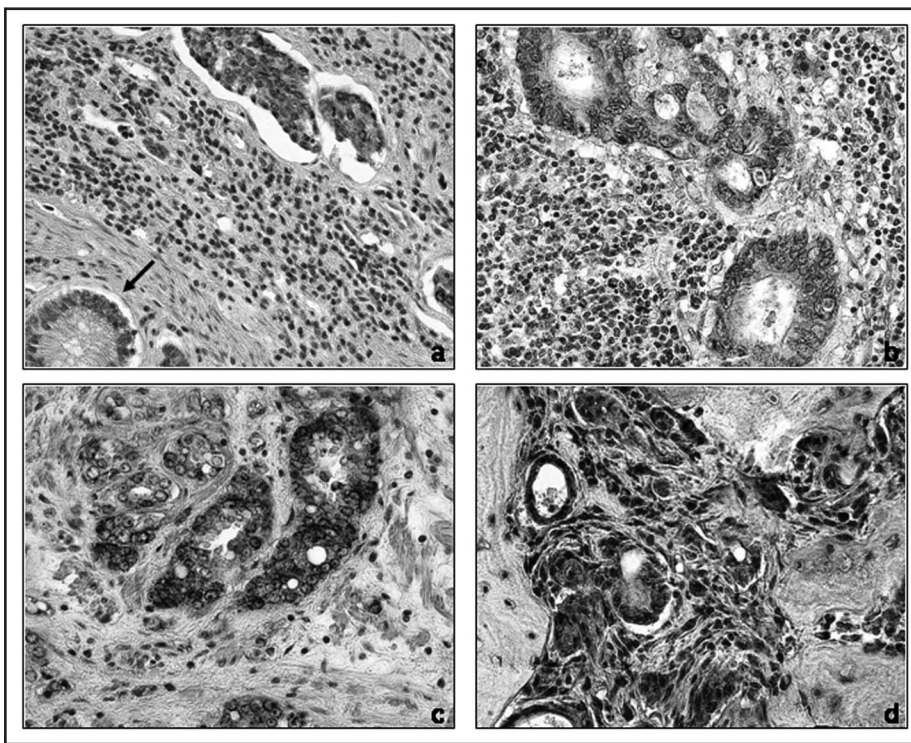
<sup>a</sup>Ref., reference.

Figure 1. Immunohistochemical detection of Hsp60 provides useful insights for histopathological diagnosis of primary and metastatic tumors. Some illustrative examples are shown here. (A) Colorectal adenocarcinoma (CRAC) is typically positive for Hsp60 (most clearly seen on top and toward the right-hand upper quadrant of the picture). In contrast, normal colonic glands are negative for Hsp60 (arrow). (B) Hsp60 immunostaining helps to identify CRAC Hsp60-positive metastatic cells in a lymph node. (C) Hsp60-positive primary adenocarcinoma cells detected in a needle biopsy specimen taken from a prostatic nodule. The same patient had bone-marrow metastases, which were also Hsp60 positive (D).

Hsp60 was scarce or absent in normal prostatic tissue.<sup>85,86</sup> High levels of Hsp60 did not correlate with an elevated Gleason's differentiation grade, indicating that determination of Hsp60 levels would not help in assessing the prognosis of prostatic cancer.<sup>86</sup>

The expression of the *hsp60* gene was found elevated in prostatic carcinoma cell lines derived from human tumors compared to normal human prostatic epithelial cells.<sup>87</sup> Interestingly, *hsp60* expression did not change significantly after heat shock, indicating that carcinogenesis was the *primum movens* of the gene's expression. The data indicate that determination of Hsp60 levels can help in the histopathological identification of malignant prostatic cells. In what regards prognosis assessment of prostatic cancer, available data are less encouraging as discussed in the first paragraph of this subsection, and because no consistent correlation was found between levels of Hsp60 in tumor specimens and outcome of primary prostatic cancers.<sup>86,88</sup>

#### Tumors of the female reproductive system.

Hsp60 was found elevated, as measured by immunohistochemistry and Western blotting, in pre-neoplastic and neoplastic lesions of the cervix.<sup>89,90</sup> The levels of Hsp60 increased from low-grade squamous intraepithelial lesions (SIL) through high-grade SIL to invasive carcinomas.<sup>90</sup>

Exposure to chronic persistent *Chlamydia trachomatis* (CT) infection has been associated with the development of ovarian and cervical cancers.<sup>91</sup> It was hypothesized that CT-Hsp60

exerts an antiapoptotic effect on tumoral cells by inhibiting caspase-3 activation, polyADP-ribose polymerase hydrolysis, and mitochondrial cytochrome-c release.<sup>91</sup> Since CT produces considerable quantities of Hsp60, determination of CT-Hsp60 could become a tool useful in the diagnosis (and possibly in assessing the prognosis) of ovarian and cervical cancers.

In ovarian carcinomas, it was shown by immunohistochemistry that high levels of Hsp60 predicted poor survival in patients undergoing chemotherapy that included cisplatin, since Hsp60 mediates drug resistance.<sup>92</sup> In another study, it was shown by immunohistochemistry that occurrence of Hsp60 was more frequent in epithelial ovarian carcinomas at the initial stage in patients that later showed a better outcome than in patients that did not do well.<sup>93</sup> Thus, in epithelial ovarian carcinomas, high levels of Hsp60 in the early stages seem to predict a good prognosis.

**Breast tumors.** Hsp60 has been found elevated by two dimensional gel electrophoresis in ductal carcinomas as compared to normal breast tissues.<sup>94</sup> In contrast, no correlation was found between levels of Hsp60 (as determined by immunohistochemistry) and tumor size, and between levels of Hsp60 and hormone receptor and HER-2 status.<sup>95</sup> Similarly, no correlation was observed between Hsp60 levels (determined by microarray analysis) and tumor stage or presence of metastases in regional lymph nodes in breast carcinomas.<sup>96</sup> In summary, the potential of determining levels of Hsp60 in the diagnosis and in assessing the prognosis of breast tumors varies depending on the method used and on tumor type.

**Tumors of the endocrine system.** Only one study has come to our attention on Hsp60 in tumors of the endocrine system. The study showed increased Hsp60 levels as determined by immunohistochemistry in adrenal Cushing tumors.<sup>97</sup> Thus, it should be explored further whether determination of Hsp60 in the adrenal is a reliable accessory tool for diagnosing Cushing tumors.

**Skin and soft tissue tumors.** Hsp60 has been investigated in skin tumors (e.g., melanoma) and in malignancies originating in mesenchymal cells (e.g., osteosarcoma and chondrosarcoma). For example, Hsp60 was found increased by proteomics and real-time PCR in cultured cells isolated from metastatic malignant melanomas as compared with cells isolated from primary melanomas.<sup>98</sup> Thus this determination deserves testing as a way to distinguish metastatic from primary melanomas.

In osteosarcomas, anti-Hsp60 antibodies were found elevated in the patients' sera at the time of first diagnosis,<sup>99</sup> and Hsp60 was also elevated in tumor specimens as measured by immunohistochemistry.<sup>100</sup> In contrast, a previous comparative study of osteosarcomas and non-malignant bone tumors did show significant differences in Hsp60 levels between the two types of specimens as measured by immunohistochemistry.<sup>101</sup> Therefore, more research is necessary to establish whether or not determination of Hsp60 levels in osteosarcomas has potential as a diagnostic/prognostic indicator, but the data available at the present time are encouraging.

The situation with cartilage tumors is still unclear. No significant differences in Hsp60 levels (as detected by immunohistochemistry) were observed between chondromas and chondrosarcomas;<sup>102</sup> thus Hsp60 determinations do not seem to offer a promising tool to distinguish these two tumors from one another.

## Hsp60 as Anti-tumor Agent and as Target for Anti-Hsp60 Compounds that Would Inhibit its Pro-Tumoral Activity

Hsp60, endogenous and overexpressed therapeutically or exogenous and administered as gene or as protein, has potential in anticancer therapy via various mechanisms.<sup>36</sup> Examples of these anti-tumor mechanisms actively involving Hsp60 are: (i) arrest of intracellular pro-survival (e.g., anti-apoptotic) pathways; (ii) stimulation of pro-apoptotic mechanisms; (iii) induction of Hsp60 surface expression and/or release into the extracellular space with activation of an antitumor immune response.

Since some Hsps are known to enhance tumor growth, the development of anti-Hsp agents for use in cancer therapy is under investigation. For instance, various classes of Hsp90 inhibitors have been developed in the last few years, and some of them are showing promising results in preclinical and clinical studies.<sup>103-105</sup> Similar efforts have not, to our knowledge, been made to develop anti-Hsp60 compounds, although these efforts seem justified at the present time, considering the new information available on Hsp60 functions and role in carcinogenesis discussed in this article.

The levels of Hsp60 can be manipulated with therapeutic purposes in various ways. Flavonoids can lower the levels of Hsp60 in a number of human tumor cell lines,<sup>106</sup> whereas cadmium induces *hsp60* gene expression in hepatoma cell lines.<sup>107</sup> In vitro experiments have shown in a variety of tumor cells that photodynamic therapy (FDT) can induce *hsp60*-gene upregulation,<sup>108,109</sup> as well as Hsp60 surface localization.<sup>110</sup> *hsp60*-gene upregulation might be responsible for the tumor-cell resistance to various forms of anti-cancer agents observed after FDT.<sup>108</sup> Intratumoral inoculation of immature dendritic cells after FDT, in in vivo experiments, resulted in an effective antitumor immune response evidenced by stimulation of the cytotoxic activity of T and NK cells.<sup>109</sup>

*hsp60*-gene silencing by small interfering RNA (siRNA) in breast and colon adenocarcinoma cells induced mitochondrial dysfunction, Hsp60-p53 complex disruption, Bax overexpression and Bax- (or caspase-) dependent apoptosis.<sup>17</sup> In contrast, siRNA-silencing of Hsp60 in normal cells did not elicit apoptosis. Thus, it would appear that knocking down or disabling Hsp60 would promote apoptosis in cancer cells only, which would make this treatment a specific killer of malignant cells, at least in breast and colon cancers.

The data summarized in the preceding paragraphs of this section are indicative of a definite potential of Hsp60 as anti-tumor agent, and as the basis of anticancer strategies aiming to promote tumor cell destruction with preservation of normal cell counterparts.

Hsp60 may also have a role as adjuvant for vaccines. DNA vaccines encoding Hsp60 linked to HPV16 E6 and E7 for human papillomaviruses-associated cervical cancer show a more potent immunotherapeutic effect than the tumor antigen without the Hsp.<sup>111</sup> Caution is in order though, since Hsp60 tolerance can be induced by repeated Hsp60 administration, which also induces cross-tolerance to other pro-inflammatory stimuli, thus potentially limiting the possible applications of this Hsp as an adjuvant or enhancer of the immune response against tumors or other pathogenic agents.<sup>112</sup>

In an experimental model, Hsp60-containing exosomes derived from heat-shocked mouse B-lymphoma cells induced a substantial antitumor CD8(+) T cell response.<sup>30</sup> The data suggest that exosome-based vaccines with Hsp60 deserve further exploration as candidates for the development of novel antitumor strategies.

Since treatment of malignant tumors usually involves chemotherapy, the impact of the latter on Hsp60 levels and functions has been investigated. For example, proteomic analysis revealed that doxorubicin does not cause significant alteration of Hsp60 levels in human breast cancer cells.<sup>113</sup> Contrarily, Hsp60 levels were notably increased after exposure of human ovarian<sup>114</sup> and cervix squamous<sup>115</sup> cancer cells to cisplatin. Since Hsp60 has a pro-survival role in these tumor cells, it could play a key role in the development of the intrinsic tumor cell resistance to anti-cancer agents induced by cisplatin.

## Concluding Remarks

Even though much has been done regarding Hsp60 roles in tumor cell survival and growth and in other cellular mechanisms, and some research has been performed concerning the use of this Hsp for oncologic diagnosis and therapy, still much more needs to be done. For instance, the presence or absence of Hsp60 in a variety of tumors has not yet been investigated so, for these tumors, it is impossible to decide whether determinations of Hsp60 will be useful for diagnostic or therapeutic purposes. Expression of the *hsp60* gene and levels of Hsp60 in relation to tumor progression and diseases prognosis have also not yet been determined for many important tumors. Furthermore, in those tumors already studied, most of the data come from immunohistochemical observations, sometimes complemented with Western blotting or proteomics, but information at the gene transcription and regulation levels is scarce. Similarly, detailed biochemical analyses of the molecular mechanisms involving Hsp60 that pertain to apoptosis and other related intracellular events relevant to tumor-cell growth and migration are limited. Likewise, the role of extracellular Hsp60 in tumor progression and in determining patient's prognosis remains for the most part to be elucidated. Further studies need to be conducted to establish the value of circulating Hsp60 as a cancer biomarker with clinical utility.

While it is true that a lot remains to be done for establishing the uses of Hsp60 determinations in oncology, the data available at this time are encouraging. It has become clear that assessing the levels of Hsp60 could be helpful in surgical pathology for a number of neoplasms, as discussed in this article. Briefs comments on some of the promising results follow.

Demonstration of Hsp60 in tumors of the digestive tract helped to: (i) distinguish dysplastic from normal tissue in tubular adenomas of the large bowel; (ii) demonstrate lymph node metastasis in colorectal adenocarcinoma; and (iii) detect vascular and neural malignant invasion.

In an illustrative case pertaining to the male reproductive tract, a bone marrow metastasis from a prostatic adenocarcinoma showed high levels of Hsp60 mimicking those of the primary tumor (Fig. 1D, unpublished data from the authors). In regard to the female reproductive tract, demonstration of Hsp60 helped to assess SIL level of exocervical dysplasia. Demonstration of low levels of Hsp60 aided in the diagnosis of bronchial, lingual and vesical carcinomas, and glioblastomas.

Another potential application of Hsp60 determinations would be in assessing prognosis and in monitoring the response to treatment. On these two areas, few studies have been reported but encouraging information has been gathered pertaining to AML, gastric MALToma and oesophageal and vesical carcinomas and, to a lesser extent, ovarian cancer.

Future goals should be to: (i) elucidate molecular mechanisms that cause up or downregulation of the *hsp60* gene during carcinogenesis; (ii) elucidate and explain mechanistically at the molecular level the effects of Hsp60 on tumor cell growth and viability, including elucidation of the Hsp60 interactions with other molecules such as those of apoptotic pathways; (iii) determine when and why during tumorigenesis Hsp60 appears on the outside of the cell membrane and/or is released into the extracellular space; (iv) use transgenic mice overexpressing Hsp60 to determine whether the incidence of cancer increases, decreases or remains unchanged, depending on the tumor type for example; (v) apply Hsp60 knockouts or knockdowns techniques to attempt to modulate tumor growth *in vivo*; (vi) establish when in the course of the disease modifications of Hsp60 levels in tumor specimens do become good diagnostic indicators; (vii) establish if, and when during the disease process, Hsp60 becomes a serum marker useful for cancer diagnosis and/or for following up disease progression and response to treatment; and (viii) use the information pertaining to the preceding seven points to design new diagnostic/prognostic methods and antitumor therapies based on Hsp60 gene or protein.

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