

Advances in the Development of Anticancer HSP-based Vaccines

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Abstract: Current advances in cancer treatment are based on the recent discoveries of molecular mechanisms of tumour maintenance. It was shown that heat shock proteins (HSPs) play a crucial role in the development of immune response against tumours. Thus, HSPs represent multifunctional agents not only with chaperone functions, but also possessing immunomodulatory properties. These properties are exploited for the development of HSP-based anticancer vaccines aimed to induce cytotoxic responses against tumours. To date, a number of strategies have been suggested to facilitate HSP-based vaccine production and to increase its effectiveness. The present review focuses on the current trend for the development of HSP-based vaccines aimed at inducing strong immunological tumour-specific responses against cancer cells of distinct etiology and localization.

Keywords: Heat shock proteins, chaperones, immune response, anticancer vaccine, tumor-associated antigens, tumor-specific antigens.

1. INTRODUCTION

Heat shock proteins (HSP) belong to a family of highly conservative proteins, members of which can be found in any living organism from bacteria to mammals. Many of these proteins are synthesized as a response to proteotoxic stress. The main functions of the HSPs are the holding and folding of intracellular proteins, and they also play an important role in the repair or elimination of wrongly folded or denatured proteins [1].

Heat shock proteins are classified by their size. There are several general families of human HSPs: small HSPs (HSPB), which include HSPs sized from 15 to 43 kDa [2], HSP40 (DNAJ), HSP60/HSP10 (HSPD/E), HSP70 (HSPA), HSP90 (HSPC) and

HSP110 (HSPH) (Table 1) [3]. They all have their own subcellular localization and similar functions aimed at maintaining the homeostasis of proteins when the cell is under stress [1].

The most-characterized HSPs are members of the HSP70 family. These HSPs are involved in a wide spectrum of processes required to maintain other proteins, such as regulation of the stress response, prevention and disruption of protein aggregates, translocation across membranes and binding to newly synthesized peptides to promote correct folding. HSP70s consist of an N-terminal ATPase domain and a C-terminal substrate-binding domain. Substrate-binding domains form a hydrophobic cavity, which can bind peptides via several types of interactions between the peptide, cavity-forming loops and the arch formed by substrate-binding loops, acting as a physical barrier to peptide dissociation [4]. Allosteric regulation of HSP70 functioning depends on its ATPase domain. "Client" protein interaction with the substrate-binding domain induces conformational change in the ATPase domain,

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Table 1. Classification of human heat shock proteins.

Family (Old/New Nomenclature)	Some Members (Old/New Nomenclature)	Intracellular Functions	Refs.
Small HSP/HSPB	HSP27/HSPB1; crystalline alpha A/HSPB4;	Promote microtubule assembly, chaperoning in folding and degradation of cytoskeletal proteins; suppressing of denaturation and aggregation, maintaining of myosin; regulates early cardiac and skeletal muscle morphogenesis.	[9-12]
HSP40/DNAJ	HSP40/DNAJB1; HSC40/DNAJB4;	Stimulation of HSP70 ATPase function.	[13]
HSP60/HSPD	HSP60/HSPD1;	Protein assembly by forming hetero-oligometric protein complex TCP1; anti-apoptotic effects.	[14]
HSP70/HSPA	HSP70/HSPA1B; HSP72/HSPA1A; HSC70/HSPA8;	Folding, refolding, blocking degradation, transporting proteins to the endoplasmic reticulum.	[15]
HSP90/HSPC	gp96/HSPC4; HSP90/HSPC1;	Helping myosin folding and sarcomere formation.	[16]
HSP110/HSPH	HSP110/HSPH2; HSP105/HSPH1.	Refolding the aggregates.	[17]

stimulating ATP hydrolysis. This, in turn, causes conformational change in the substrate-binding domain, closing of the substrate-binding cavity and holding of the protein. Thus, the affinity of HSP70 for client proteins depends on the state of the ATPase domain: affinity is high in the ADP-bound state and low in the ATP-bound state [5]. Moreover, the ability of HSP70 to bind receptors onto the surfaces of cells is also partly regulated by the ATPase domain [6].

HSPs not only perform the protein-maintaining function inside the cell but they are also considered to have the extracellular ability to promote cell-cell interaction as cytokines. HSP-peptide complexes can be released into the extracellular milieu from disintegrated cells under necrosis. Moreover, HSP70 could be secreted during intensive stress or in basal conditions from intact cells [7]. Thus, it was supposed that HSPs carry some extracellular functions apart from the fact that they are chaperones. Indeed, HSPs have been observed to interact with a number of immune cells via several receptors, and therefore immunomodulatory properties were attributed to HSPs [8]. In this process, the heat shock proteins chaperone tumour antigens as well as normal proteins, and serve as carriers to antigen-presenting cells (APCs). Studies on HSPs over the past two decades have provided important information for understanding the mechanisms of such antitumour immune responses.

Anticancer vaccination is very attractive for use in cancer immunotherapy. Anticancer vaccines are relatively safe and show no toxic effects compared with traditionally used chemotherapy and radiotherapy [18]. However, administering cancer-specific peptides alone raises different problems, as they are less immunogenic than those of viral or bacterial origin. Thus, adjuvants that will increase the immunogenicity of vaccines and the quantity of peptides taken up by APC are required. Different approaches have been suggested to improve cancer vaccines, such as oil adjuvants and including recently applied self-assembling synthetic peptides [19]. Compared to these, HSP vaccines are more attractive to use because they have a natural ability to interact with APC and other immune cells. However, HSP vaccines have a wide range of efficacy, which is evidenced not only by the immunosuppression of the tumour micro-environment, but also by vaccine production methods, the type of tumour-associated antigens used and the patient's stage of disease, in the case of autologous HSP vaccines. This review focuses on discussing the excellent potential of HSP-based vaccines and the current trends of their improvement.

2. HEAT SHOCK PROTEINS, IMMUNOMODULATION AND CANCER RESPONSE

The detection of HSPs in the extracellular environment suggested that HSPs have other functions besides

being intracellular chaperones. Indeed, subsequent studies have revealed the immunological properties of heat shock proteins. Pramod Srivastava was one of the pioneers of the novel HSPs immunomodulatory functions discovery, and he reported the ability of HSP70-peptide complexes to increase antitumour immunity, and thus showed that HSP70 could act as an antigen carrier [20]. Later, this increase in immunity was attributed to the cross-presentation of the delivered peptide [21]. As chaperones, HSPs interact with proteins in equal quantities (stoichiometric), and therefore enormous quantities of HSPs are synthesized during cell stress to form complexes with cell proteins and to chaperone them. Cells affected by viruses and bacteria, or malignant cells also experience stress that eventually could lead to cell necrosis accompanied with HSP-peptide complexes release into the extracellular space. Moreover, cancer transformation means that cells are already under cellular stress conditions. Thus, HSP levels in cancer cells are higher than in normal cells [22, 23]. This situation occurs due to the high expression of mutated and misfolded cancer-specific proteins,

oxidative stress and the lack of nutrients in cancer cells. High levels of mutated proteins that corrupt the processes of cell proliferation, antigrowth signalling and cell death also trigger high-level expression of HSPs to chaperone them. Thus, intracellular HSPs play the role of cancer promoters by stabilizing the altered conformation of mutant proteins that drive carcinogenesis [24].

Considering the highly stressed tumour milieu, this could lead to HSPs' release from cancer cells via necrotic disintegration. There are many immune cells in the area surrounding necrosis. It was observed that HSP-peptide complexes, including complexes with exogenous viral, bacterial or tumoural peptides, could be engulfed by APC via endocytosis after entering the extracellular environment (Fig. 1) [6]. Which receptors are responsible for HSPs' internalization by APC is a question still under discussion. However, recent studies have shown that scavenger receptors could be involved in this process.

It was established that a subgroup of scavenger receptors, including LOX-1 and FEEL-1, could bind

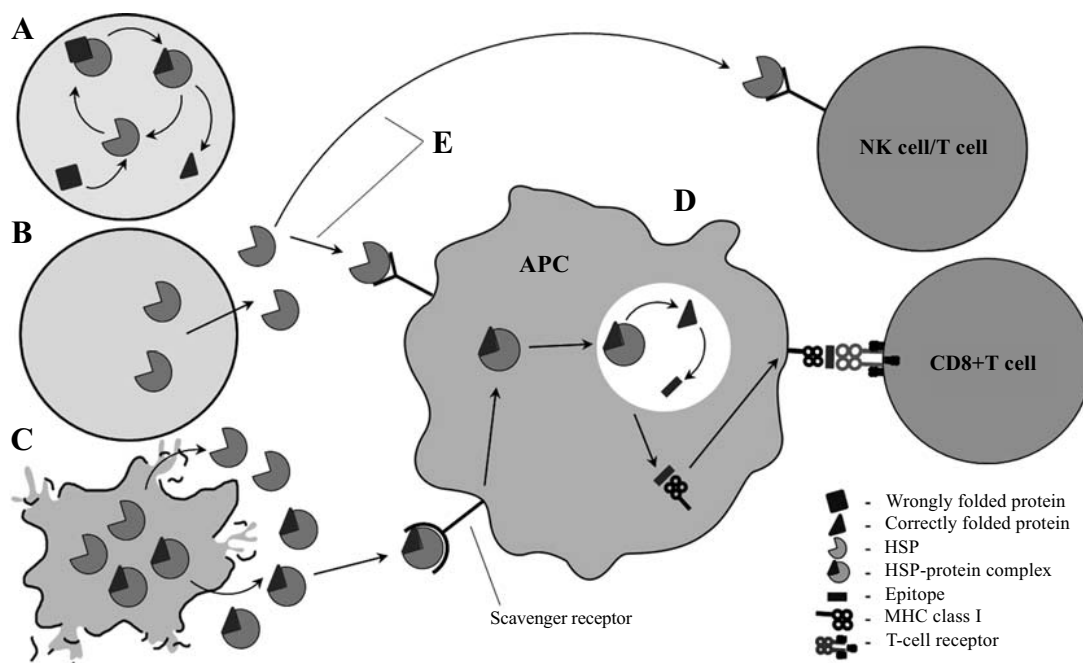


Fig. (1). Interaction of extracellular HSPs with immune cells.

(A) Protein maintaining function of intracellular HSP. HSPs interact with misfolded proteins via peptide-binding domain, providing holding and correct folding. (B) HSPs release from intact cell. HSPs may be released in three different forms from intact cells: in a free form, in the membrane-associated form or within the exosomes. (C) HSPs release from necrotic cell. Cells, being disintegrated, are the major source of extracellular HSPs. HSPs may be released from necrotic cells both, in free form and in complexes with cellular proteins. (D) Cross-presentation of protein, delivered by HSP. HSP-peptide complexes are engulfed by APC after binding to scavenger receptors. After internalization, complexes are involved into the cross-presentation resulting in activation of CD8+ T cells epitope-specific cytotoxic responses. (E) Free HSPs mediate immunomodulatory effects in distinct immune cells.

mammalian HSP70 as well as recombinant HSP70, except for SREC-1 which could bind mammalian HSP70 well but recombinant HSP70 only poorly [6].

After binding, these receptors mediate the uptake of HSP70-peptide complexes into APC. In this way, the captured peptides will be processed by APC and involved in cross-presentation. After the processing of antigens, the epitopes in the complex with MHC class I and class II will be presented to T cells [25]. These will activate T killers to cause a cytotoxic response and T helpers that will, in turn, activate B cells to cause a humoral response.

Nevertheless, it is not only the antigen-specific T-cell response that could be mediated by HSPs. Evidence suggests, that HSPs mediate other immunomodulatory effects also. The major source of extracellular HSPs is necrotic cells [26]. However, HSPs could also be released from intact cells in different ways. Indeed, it was confirmed that HSPs were released from tumour cells in special non-classical ways [27, 28]. Effects that cells exhibit after interaction with HSPs depend on the way HSPs are secreted. HSPs are known to be secreted in free form, in membrane-associated form and in exosomes [7, 28, 29]. Such secreted HSPs could have either immunosuppressive or immunostimulatory properties [30, 31] (Table 2).

There have been several experiments to reproduce the modulatory activity of free-form secreted HSPs. HSP70 was observed to downregulate the production of IL-6, IL-8 and MCP-1 induced by TNF- α [32]. In addition, a reduction of DC maturation was observed if treated with HSP70 [33]. Also, no cytokine secretion from DC was detected [43]. Moreover, it was observed that besides the absence of mature DC to stimulate CTL, HSP70 could reduce T-cell response independently of the stimulatory effects of DC on these cells [33].

HSP60 secreted from cells mediates the increasing levels of CD4, CD25 and Foxp3 cells, leading to a suppression of CTL [37]. In addition, HSP60 stimulates mononuclear cells to induce the production of cytokines such as IL-10 and IL-6 through upregulation of CD4⁺ T cells [38]. CD4⁺ T cells stimulated in this way by mononuclear cells exhibit immunosuppressive properties after their proliferation. B cells were also observed to be induced by HSP60 treatment. Such B-cell activation was shown to be possible via TLR4, and the response leads to B-cell proliferation and IL-10 and IL-6 production [39]. On the other hand, whereas B cells start to produce anti-inflammatory cytokines after HSP60 treatment, they simultaneously become capable

of stimulating T cells to secrete TNF- α as well as IL-10 [39]. Finally, the ability of HSP60 to induce TNF- α production by macrophages was demonstrated [40]. Interestingly, HSP60 overexpression in tumour cells promotes cancer not only via its extracellular immune properties that contribute to the immunosuppression of the tumour milieu, but also via its chaperone functions. It was reported that overexpressed HSP60 could promote metastasis through interactions with β -catenin, increasing its protein levels and enhancing its transcriptional activity [45].

In the case of HSP27, it was reported that it has the capability of inducing the differentiation of monocytes to immune-tolerant phenotype macrophages that begin to produce anti-inflammatory mediators such as thrombospondin-1 and IL-10, and that have an influence on T cells, leading to their anergy [23, 34]. Moreover, these macrophages become extremely pro-angiogenic and will provide neovascularization, which is one of the keys to tumour progression [23].

Exosome-dependent mechanisms of HSPs secretion also make their contribution to immunosuppression. HSP72-positive exosomes were observed to attract myeloid-derived suppressor cells (MDSCs) and to trigger a Stat3-dependent pathway of MDSCs suppressive activity determination [46]. MDSCs in turn mediate immunosuppression by secreting IL-10, decreasing CD4⁺ and CD8⁺ T-cell viability and attracting Treg cells [47]. However, the exosome-associated secretion of HSPs could act as an immunostimulatory response together with immunosuppression. Such HSP70 surface-positive exosomes are able to induce the specific migration of NK cells and to boost their cytolytic function [41]. In this case, NK cells will be enhanced to release perforin and granzyme B, which will specifically bind with the target cells of natural killers and lead to target-cell apoptosis. Other immunostimulatory effects of HSPs were observed if associated with a plasma membrane. HSP70 could appear in the lipid bilayer of cells after stress and remain in a membrane-bound state or be released in a membrane-associated form [29]. If released in membrane-associated form it stimulates TNF- α production by activating macrophages. Moreover, the membrane-associated form of HSP70 is able to activate both the TNF- α production of macrophages and the cytolytic activity of NK cells [29, 42].

Thus, heat shock proteins were investigated as one of the most important immune-response participants. It is widely known that tumour genesis and immune-

Table 2. Immune functions of extracellular heat shock proteins.

Name	Immunostimulatory Properties	Immunosuppressive Properties	Refs.
HSP27		Induction of monocytes differentiation to immune-tolerogenic macrophages; increase IL-10 release by macrophages	[23, 34-36]
HSP60	T-cell IFN γ secretion mediated by HSP60-activated B-cells; induction of TNF- α production by macrophages	Induction and upregulation of Tregs; induction of IL-6 and IL-10 production by B-cells	[37-40]
HSP70	Transfer of antigens/peptides to APC followed by involvement into cross-presentation; induction of migration and cytotoxic activity of NK-cells; stimulation of TNF- α production by macrophages	Downregulation of proinflammatory cytokines production; reduction of T-cell response and DC maturation	[20, 29, 32, 33, 41-43]
HSP90	Transfer of antigens/peptides to APC followed by involvement into cross-presentation		[31]
HSP110	Transfer of antigens/peptides to APC followed by involvement into cross-presentation		[44]

response modulation are inseparable. HSPs, as immune-response participants, are no exception, and they also play a significant role in tumour genesis. It seems that HSPs tend to have two balancing roles in cancer. Their immunosuppression abilities greatly contribute to cancer promotion, creating an immunosuppressed tumour micro-environment, which leads to immune cells' tolerance and anergy, allowing the tumour to avoid immune response. Such extracellular effects, along with HSPs' intracellular cancer promotion effects, make HSPs one of the important tumour progression factors. On the other hand, HSPs could induce antitumour response and show immunostimulatory abilities such as the activation of NK cells and T cells via cross-presentation of tumour antigens by APCs. It has been observed that such situations present an opportunity for immune attack on tumour cells. One possible way to achieve this is to activate the antigen-specific cytotoxic antitumour response using the mechanism described earlier, i.e., CD8⁺ T-cell activation through APCs mediated by heat shock proteins.

3. TUMOR SPECIFIC HSB-BASED VACCINES

Srivastava and colleagues were the first to recognize heat shock proteins as immune regulatory molecules. Their discovery of the potential function of HSPs in regulating the immune response gave rise to numerous studies in this field. As a starting point, there was the report that recognized gp96 as a tumour antigen carrier. Initially recognized as a tumour-specific antigen (TSA) [48], gp96 was then reported to be a transporter for TSAs, due to its well-conserved structure [49]. It was observed that gp96, in association with tumour antigens, could elicit specific immunity to the tumours

from which they were isolated. Later, the same ability to enhance antitumour immunity when associated with tumour peptides was shown for HSP70 and HSP90 [20, 50]. The mechanism for such an increase in immunity was assigned to cross-presentation [21]. Therefore, HSPs would have to be associated with MHC class I epitopes to activate CTL after their cross-presentation. Indeed, the isolation and analysis of peptides associated with HSP70, HSP90 and gp96 revealed that these peptides were MHC class I epitopes of tumour antigens [51]. Moreover, the precursors of such epitopes could also form complexes with HSPs. Later, it was observed that not only CD8⁺ T cells could be activated via this mechanism, but also CD4⁺ T cells. HSPs could also associate with MHC class II epitope precursors of tumour antigens that could be cross-presented for activation of CD4⁺ T cells [25]. All these findings were understood by the scientific community, who began to undertake studies aimed at adapting HSPs for cancer treatment.

As described, the main idea was to use HSP-peptide complexes as vaccines to activate antitumour immunity via HSP-delivered tumour peptides cross-presentation. There has been a proposal to use tumour-derived autologous HSP-peptide complexes as antitumour vaccines. The autologous preparations of tumour-derived HSP-peptide complexes were shown to be effective in limiting tumour growth and metastasis in mice [52]. It should be noted that preparations isolated from synergetic, but not autologous tumour showed no significant efficacy. This is explained by the fact that each malignant formation has its own unique proteome. Such a preparation of tumour-derived HSP-peptide complexes

could form a polyvalent vaccine that could provide cross-presentation of a full palette of antigens of the tumour, from which the complexes were isolated. Thus, autologous HSP vaccines must be tailored to each patient and their own malignant formation with its unique proteome. Later, it was observed that melanoma-derived HSP70-peptide complexes could be cross-presented by human DCs, which in turn could stimulate peptide-specific T-cell response [53]. It was also found that CD8⁺ T cells require no additional DC signals to be stimulated, except cross-presentation of MHC class I epitopes.

Due to the features discovered, tumour-derived HSP-peptide complexes have been used as promising individual antitumour vaccines. There have been several clinical trials that have demonstrated some autologous HSP vaccines efficiency in the treatment of such cancers as melanoma [18], colorectal cancer [54] and renal cell carcinoma with vitespen [55]. HSP autologous vaccines extracted from tumours have been proven to be safe and effective, giving personalized therapy for patients against some cancers [18]. However, these methods have their disadvantages and restrictions. One significant limitation is the low yield of patient tumour tissue. A large tumour tissue volume is required in order to harvest enough HSP-peptide complexes. About half of the patients excluded from trials had an insufficient amount of tumour tissue to prepare the vaccine [18, 55]. Moreover, a large volume of tumour tissue usually correlates with a late stage of cancer, when such therapeutic methods will be useless. With regard to vitespen, which is a kind of anticancer therapeutic agent that consists of gp96-peptide complexes purified from the individual patient's tumours, this only shows significant effects in treating early-stage renal cell carcinoma [55]. Vitespen has failed to show broader activity in randomized clinical trials for treating the advanced stages of such diseases as glioblastoma, colorectal cancer, non-Hodgkin's lymphoma, pancreatic cancer, non-small cell lung cancer and gastric cancer [56]. It had encouraging results in only a few selected patients. Accordingly, the best strategy for applying autologous HSP vaccines is to administer it preventively, or to administer it in the early stages of cancer. Another possibility is to use HSP vaccines as preventive treatment against recurrence after tumour resection. However, disadvantages include the fact that the harvesting of HSP-peptide complexes may be limited due to tumour heterogeneity, as tumours could contain connective and vascular tissue with large areas of necrosis and immune cells infiltrates. Moreover, the transcriptome and proteome of

the original tumour cells and those of their metastasis could be different [57, 58].

An alternative possibility is to use *in vitro* constructed complexes of HSPs with tumour-associated antigens (TAA) or their peptides. This idea appeared at about the same time as the idea of autologous vaccines. The feasibility of HSP-peptide complexation *in vitro* was shown in an example with HSC70 and HSPA5 chaperones [59]. Tumour-associated peptides were no exception and were also shown to bind with HSP70 and gp96 *in vitro* [21, 60]. Such reconstructed complexes were observed to be able to elicit antitumour immunity and CD8⁺ T-cell cytotoxic response in the same manner, by being taken up by APCs as autologous preparations [21]. Since that time, there have been attempts to create HSP vaccines *in vitro*. Such methods of *in vitro* complex reconstitution have several advantages compared with autologous vaccines, one of them being the possibility of avoiding the restriction of the patient's tumour volume. For this reason, questions about insufficient amounts of preparation are disappearing. Binding whichever tumour antigen peptide is needed could solve another problem, i.e., that of the different proteomes of primary tumour and metastasis. Such an opportunity could also solve the problem of the personalization of HSP-based antitumour vaccines. Nevertheless, the opportunity of *in vitro* vaccine production does raise another problem. It is impossible to synthesize all antigens represented in the tumour proteome in order to make complexes with HSPs *in vitro*. The only method for achieving this is to extract them from the patient's tumour, but this procedure is little different from the case of HSP-peptide complexes extraction and faces the same problems. Moreover, the vaccine production process is complicated by the subsequent stage of *in vitro* re-complexation. Thus, it is necessary to study the tumour proteome to reveal tumour-associated antigens, epitopes of which will provide strongly specific and effective antitumour response. It would be sufficient to use one or several antigenic epitopes as a vaccine against one type of tumour, rather than the whole tumour proteome, as for autologous vaccines. A search for antigens and their epitopes that are more immunogenic and specific for malignant cells is needed, in order to achieve the great potential of HSP-based vaccines.

4. TUMOR-ASSOCIATED ANTIGENS AS A POTENTIAL COMPONENT OF ANTICANCER VACCINES

The malignant transformation of normal cells is accompanied by dramatic rearrangements in the structure

and function of their molecular machinery. In particular, the protein expression patterns undergo substantial changes due to up- or downregulation of the synthesis of certain proteins. Furthermore, upon malignant transformation, normal cells frequently start to produce a protein or even a set of proteins, which are foreign to the specific type of cells within a certain tissue [61]. These proteins, specifically expressed by tumours, are designated as tumour-associated antigens (TAAs). Based on their origin these antigens can be divided into two major categories: antigens of high tumoural specificity and antigens of low tumoural specificity [62]. In turn, these two categories each include several groups of antigens (Table 3). The accumulation of TAAs immunologically distinguishes tumour cells from normal cells, and therefore naturally occurring antitumour immunity can be activated by amplification, overexpression or aberrant ectopic expression of the proteins possessing antigenic properties.

4.1. Low Tumoural Specificity Antigens

The antigens of low tumoural specificity include antigens that are expressed not only in tumour cells, but also in normal cells of the original tissues. There are two groups of antigens in this category: tissue-specific or differentiation antigens and overexpression antigens. Differentiation antigens are a type of antigen that can only be synthesized in tumours or in normal cells from which the tumour cells originated. To date, several antigens of normal tissues have been described as differentiation antigens. NY-BR-1 antigen was reported to be synthesized in normal breast tissue and in breast cancer cells [72, 73]. No other normal tissues or tumours were observed to express NY-BR-1, except normal testis tissues. The tissue-restricted antigens tastin and bystin were also reported to be differentiation antigens, whose expression could be observed in normal placental and testis cells, as well as in epithelial ovarian carcinoma cells [74]. Myeloid cell nuclear differentiation antigen, shown to be expressed in normal myelomonocytic and B cells, as well as in B cell lymphomas, could thus be assigned to the group of differentiation antigens [75]. The most-studied differentiation antigens are melanocyte-specific antigens. These include tyrosinase [76], gp100 [77] and Melan-A/MART-1 [78], which can be expressed by both normal melanocytes and melanoma cells. It appears that immune-system tolerance for differentiation antigens is not absolute. T-cell response against these proteins could spontaneously appear in patients with cancer [76, 79]. Thus, the question of using differentiation antigens or their peptides as an anti-tumor vaccine component is controversial, due to their

low specificity for tumour cells and potential ability to enhance immune response against normal cells which are also expressing such proteins.

Some normally expressed proteins, if overexpressed in tumour cells, could elicit specific T-cell response against the tumour cells. Usually, T cells do not recognize self-cells due to their low affinity with HLA-peptide complexes whose peptides are from normally expressed proteins. In the case of protein overexpression above some threshold, this will also lead to overexpression of HLA-peptide complexes on the surface of the cell. This high density of HLA-peptide complexes will compensate for their low affinity for T cells, making specific T-cell recognition of these cells possible [80]. Thus, overexpression antigens could be a possible target for anticancer vaccines. The most-studied antigen of this group is HER2, also known as ERBB2 and NEU. HER2 has been intensively studied in breast and ovarian cancer, where its overexpression is detected in 30-40% of cases of both cancers [81]. HER2 has also been reported to be overexpressed in other cancers of epithelial origin such as gastric cancer [82] and colorectal cancer [83]. Several antigenic peptides of HER2 have already been identified as potential components of vaccines [80, 84]. However, the application of such anti-HER2 vaccines will face problems such as immunological tolerance against HER2 protein [85]. Thus, the problem of how to break such tolerance is restricting the future application of HER2 as an anti-cancer vaccine component.

4.2. High Tumoural Specificity Antigens

The second major category of TAAs is that of antigens of high tumoural specificity, which includes antigens that are a result of mutations or genome rearrangements, antigens of oncogenic viruses and tumour-specific antigens that are a result of aberrant expression of non-specific tissue genes. Being strictly specific for tumour cells, vaccines with these antigens could elicit specific immune responses against tumour cells, which is key to immunotherapy for cancer.

Mutations, depending on their type, lead to the changing of the normal protein sequence by changing an amino acid, either by frameshift or by inserting a stop codon. This situation will cause the generation of a new antigen peptide sequence in place of the original sequence. The modified protein will be recognized by immune cells as foreign, and specific T-cell response will be developed. There are several examples of mutated antigens that belong to this group of TAAs: CDK-4 [86], β -catenin [87], mutated p53 [88] and MUM-1

Table 3. Tumour-associated antigens.

	Group	Examples of Antigens/Peptides Used in HSP-based Vaccines		Refs.
		Antigen/Peptide	Type of HSP Vaccine	
Low tumoural specificity anti-gens	<i>Tissue-specific (differentiation) antigens</i> Subgroup: Melanocyte-specific antigens Prostate-specific antigens Breast-specific antigens Ovarian-specific antigens	PSCA	HSPH4/Grp170 complexes	[63]
		GnRH	HSP65 fusion protein	[64]
		MART-1	HSPC4/gp96 complexes	[65]
	<i>Overexpression antigens</i>	Heparanase	HSP110 complexes	[66]
High tumoural specificity anti-gens	<i>Mutated antigens</i>			
	<i>Viral antigens</i>	E7 of HPV16	HSP70 fusion protein	[67]
		E7:49-57 peptide of HPV 16	HSP110 complexes	[68]
	<i>Tumour-specific antigens</i> Subgroup: Cancer-germline antigens Onconeural antigens Cancer-retinal antigens	NY-ESO-1:157-165 peptide	HSC70 fusion protein	[69]
		TRP2:175-192 peptide	HSP110 complexes, HSPH4/Grp170 complexes	[70]
		MAGE-1	HSP70 complexes	[71]

[89]. It should also be noted that TAAs of this group usually drive oncogenesis in the process of cell malignantization. The variety of possible mutations or genome rearrangements and resulting peptide sequences is vast; therefore, peptides that are the result of such mutations are not likely to be useful as components of antitumour vaccines for mass use.

Oncogenic viruses are known for their contribution to tumour development in several cancer diseases. There are several viruses associated with a subset of tumours: human papilloma virus (HPV) (cervical cancer), Epstein-Barr virus (Hodgkin's disease, nasopharyngeal carcinoma, Burkitt's lymphoma) and hepatitis B or C viruses (hepatocellular carcinoma) [61]. In the case of retroviruses, on being transfected into cells, they transform the cell genome with oncogene. This situation will lead to viral antigen (oncoprotein) expression from oncogene. In the case of DNA-containing viruses, viral antigen expression will occur from viral DNA-containing oncogene without cell transformation. Both situations cause the synthesis of viral antigens foreign for immune cells that could activate antigen-specific T-cell response against tumour cells transfected with oncogenic viruses. Thus, viral antigens are suitable candidates for antitumour (HSP-based) vaccine components. Due to their very early expression, before the onset of malignant transformation, these vaccines, containing viral oncogenes, could be more promising for preventive vaccination than for therapeutic purposes [90]. Indeed, clinical trials reported highly efficacious prophylactic properties of

HPV antigen vaccine against both HPV-associated cervical cancer and HPV infection [91].

Tumour-specific antigens (TSAs) along with the rest of the TAAs are possibly the most suitable tumour antigens for anticancer vaccines. TSAs, which are usually expressed in cells that have a lack of HLA molecules or those located in immunoprivileged tissues, become foreign to immune cells if they are aberrantly expressed in tumours. Thus, a strictly specific T-cell response against tumour cells could arise. Cancer-germline (testis) antigens were the first to be recognized as TSAs. They were first found to be expressed in melanoma cells, whose genes were named the melanoma-associated antigen family (MAGE) [92]. To date, the MAGE family includes 25 genes, expressed in a wide range of tumours, not only in melanoma. The whole cancer-germline group of TSAs contains more than 60 antigens and consists of several antigenic families, including MAGE, BAGE, CAGE, SSX and CTAG [62]. The expression of cancer-germline antigens was not found in any normal tissues except germ cells and trophoblastic cells. The anergy of the immune system to these cells is explained by the lack of MHC class I molecules on germ cells, and therefore, the germ cells could not elicit T-cell response [93]. The blood-testis barrier also contributes to this property [94]. A large percentage of tumours of a particular type could aberrantly express cancer-germline genes [95]. Thus, products of these genes, being foreign to immune cells, result in their host tumour cells being specific targets for T-cell response.

Other groups of TSAs are onconeural antigens and, as a subcase, cancer-retinal antigens (CRAs), which were recently reported as a new group of tumour antigens [96, 97]. Onconeural antigens and CRAs are normally only expressed in the nervous system and retina respectively, which are immunoprivileged zones due to the blood-brain and blood-retinal barriers. During the process of cell malignization these antigens, as well as the cancer-germline antigens, can begin to express aberrantly. The presence of tissue barriers makes onconeural antigens and CRAs foreign for the immune system, and thus, being expressed in tumours, they could be cross-presented for T cells followed by T-cell specific response. Due to the tumour-specific expression of onconeural antigens and CRAs, except for immunologically privileged nerve and retinal cells, it makes sense to refer to them as tumour-specific antigens with respect to the classification used above. However, there is a significant distinction between cancer-germline and both onconeural and cancer-retinal antigen groups. Nerve cells, as well as retinal cells, can express MHC class I molecules, whereas germ cells lack this ability, which makes them invisible to immune cells [98]. The blood-brain barrier is known as a natural hedge that strictly controls the passage of drugs and microorganisms, as well as antibodies and lymphocytes [99]. Nevertheless, in some cases it can be permeable [100]. Thus, the autoimmune response against nerve and retinal cells could appear. Clinical cases of paraneoplastic syndromes (PNS) could confirm these situations. PNSs arise as a consequence of cancer but they are not associated with tumour tissue as such. They are characterized by the manifestation of extra-tumour symptoms that appear due to the tumour's production of certain substances. The pathomechanisms of PNS have been well studied in paraneoplastic retinopathy or cancer-associated retinopathy (CAR), which is a type of PNS that includes blurred vision, complaints of flashing lights, loss of peripheral and colour vision and night blindness [101]. These symptoms occur due to the autoimmune response against host retinal cells after the recognition of aberrantly expressed cancer-retina antigens by tumour cells. In this case, autoimmunity is mediated by T- and B-cell response, supported by the production of antibodies against CRAs [102, 103]. However, the activation of immune response against CRAs is often not manifested. The proportion of cancer patients with detected CRA autoantibodies is greater than that of patients who have developed cancer-associated retinopathy [104]. One of several hypotheses explaining such a situation is that the titre of anti-CRA antibodies must exceed a

certain level to pass through the blood-brain barrier [105]. This statement is supported by the report that only patients with a high titre of anti-Hu and anti-recoverin antibodies showed PNS [106]. It should also be noted that anti-CRA antibodies could be detected long before the cancer can be diagnosed by the usual diagnostic techniques, meaning that malignization is already ongoing somewhere [107].

To date, there are several proteins known as “classical” onconeural antigens: HuD, Yo, CRMP5, amphiphysin, Ri and Ma2 [108], with the HuD antigen being the most extensively studied amongst them. HuD antigens were observed to express in an overwhelming proportion of small cell lung cancer and neuroblastoma cases [109]. As for other onconeural proteins, their aberrant expression is most common for lung cancer, breast cancer and ovarian cancer [108, 110, 111].

Recoverin was first recognized as a cancer-retina antigen [112]. Several cell lines and tumours were reported to express recoverin [113, 114]. More retina-associated proteins were shown to be aberrantly expressed in tumours, such as rhodopsin, transducin, PDE 6, rhodopsin kinase and arrestin [115]. These proteins were observed to be expressed in several tumours [108, 116]. It is interesting that recoverin was reported to be expressed mostly in the early stage of cancer for gastric cancer [117]. The mechanism, due to the aberrant expression of cancer-germline antigens and, in at least one cancer-retina antigen, recoverin occurs, is the same [118, 119]. The demethylation of the gene promoter has been described as such a mechanism. Cancer-germline genes and recoverin gene promoters are methylated in normal cells, except in germ and retina cells, leading to their silencing. The rearrangements of the molecular machinery during the malignization process are the reason for the increased levels of demethylation agents that cause demethylation of the genes' promoters, and therefore, their enhancement.

Thus, considering features of onconeural and cancer-retina antigens, such as tumour-specific aberrant expression and their appearance long before cancer manifestation, they could be used as components of HSP-based antitumour vaccines for preventive purposes or for early-stage treatment. However, it is necessary to be cautious due to MHC presence on normally onconeural antigen-expressing and CRA-expressing cells, and therefore, the possibility of autoimmune response activation against them. One possible solution is to avoid MHC class II epitopes as vaccine components to prevent humoral response and antibody production. Although cancer-germline, onconeural and

cancer-retinal antigens were shown to express in several tumours, each candidate patient must be tested for the TSA's tumour expression, for rational use of the anticancer vaccine. It makes sense to assess patients for variants of HLA class I and II molecules. This knowledge will allow one to find suitable epitopes of antigens with high affinity with the patient's HLA variants.

Overall, various histohaematic barriers have been described: the blood-retina barrier, blood-brain barrier, blood-testis barrier, blood-placenta barrier and blood-tendon barrier. Thus, several of the more immunologically privileged zones could contain more cells, the protein products of which will be foreign for the immune system. Such foreign proteins could be potential tumour-specific antigens.

It has become clear that not only adjuvants are responsible for vaccine efficiency, but also well-selected peptides or epitopes of highly specific tumour antigens. The affinity of antigenic epitopes to the patient's MHC molecules also plays one of the most important roles in immunogenicity. Two different epitopes of the same antigen, cross-presented by APC, could show different abilities for eliciting antigen-specific T-cell response against tumour cells which express this antigen [120]. However, administering peptides alone might not be sufficient for strong immunogenicity. Indeed, it was reported that the NY-ESO-1 epitope, known as a highly specific cancer-germline antigen, showed much more efficient results in CTL response activation *in vitro* when fused to HSC70 than NY-ESO-1 alone [69]. Thus, vaccines that include HSPs as adjuvants for peptide delivery into APCs followed by efficient cross-presentation, and that also include well-selected tumour-specific antigenic epitopes, could be a promising therapeutic agent for preventing or treating cancer.

5. PEPTIDE-SPECIFIC HSB-BASED VACCINES

5.1. HSP-peptide Complexes

Information received from studies on the tumour proteome showed that tumours are immunologically distinguished from normal cells, and also that proteomes of different types of tumours are different. This occurs because each tumour specifically expresses TAAs. Therefore, using epitopes of TAAs as vaccine components gives an opportunity to create an anti-cancer vaccine that will elicit specific immune response (Table 3). Due to the fact that only one or several TAAs are needed for this, it has become possible to produce such a vaccine *in vitro* from recombinant proteins, instead of extracting tumor proteins as in autologous vaccine production. Thus, different varia-

tions of reconstituted *in vitro* HSP-peptide complexes were prepared and offered as antitumour vaccines. There are a number of studies with similar results that showed the efficacy of reconstituted *in vitro* complexes of HSPs with a single peptide of tumour or microorganism antigen [65, 121]. Not all the proteins, but only a subset, could bind HSPs with suitable affinity. Hybrid peptides were prepared to prevent such problems in these complexes [122]. The peptides consisted of a known MHC class I epitope of the antigen, which was joined to a peptide known as a high-affinity ligand for HSP70 by a glycine-serine-glycine linker. It was observed that HSP70 complexes with hybrid peptides demonstrated higher efficacy than HSP70 complexes with peptides without a ligand for a chaperone. It should be noted that the HSP70 used was recombinant, but not isolated from mammalian cells. Another study with a higher-affinity ligand for HSP70 and a cleavable linker confirmed the statement that the high affinity of HSPs for the peptide is crucial for the ability of complexes to elicit an immune response [123]. Moreover, the higher the affinity for HSP, the higher the achievable immunogenicity [123]. A peptide is more likely to be delivered to APC if the interaction time with HSP is longer. Indeed, hybrid peptides with a higher-affinity ligand to HSP70 showed higher immunogenic activity than those with lower-affinity ligands. Thus, this observation also allows us to confirm that a lower dose of HSP-peptide complexes with high affinity can elicit the same response as at higher dose of HSP-peptide complexes with lower affinity [123]. In addition, multiple-peptide complexes with HSP70 via a more cleavable linker were created. Such multiple-peptide constructions allow more than one epitope to be delivered in a single immunization, which offers an opportunity to construct polyvalent-type vaccines [123]. Another study provided interesting data suggesting that truncated HSP70, but not the whole protein, is sufficient to cause CTL response. The 136-aminoacid peptide-binding domain of HSP70 alone was still capable of inducing peptide-specific CD8⁺ T-cell response [124].

Overall, *in vitro*-constructed complexes of HSP with tumour antigen peptides were shown to be effective in eliciting CTL peptide-specific response. However, there is still controversy over which type of HSP-based vaccine is more desirable (autologous or reconstituted *in vitro*) [125]. An advantage of autologous HSP vaccine is its polyvalence, meaning that such vaccines could provide the whole spectrum of tumour antigens. This hypothesis is confirmed by several studies of multiple-peptide vaccines that showed greater efficacy against infectious and cancer diseases than single-

peptide agents [126, 127]. Nevertheless, many examples could be listed that showed higher efficacy of single-antigen vaccines compared with multi-antigen cocktails [128].

5.2. HSP-peptide Fusion Proteins

Developing the idea of *in vitro*-reconstituted HSP-peptide complexes as antigen-specific vaccines, there was a proposal to use HSP-peptide fusion proteins (Table 3) Suzue *et al* first reported the concept of such constructions [129]. Recombinant fusion protein of *M. tuberculosis* HSP70 with 115-aminoacid ovalbumin peptide attached to the N-terminus of the chaperone was synthesized and isolated from *E. coli*. It was observed that the HSP70-ovalbumin peptide fusion protein stimulated the production of CD8⁺ T cells that recognized ovalbumin-expressing melanoma tumour cells [129]. However, More *et al.* reported that a fusion protein of gp96 with CTL epitope, fused to the C-terminal of gp96 without linkers, failed to show any significant activation of epitope-specific T cells [130]. Nevertheless, it was poorly understood why the failure of gp96, compared with mycobacterial HSP70, occurred. Among the possible explanations for this was the hypothesis that conformational changes due to covalent peptide binding to gp96, and C- and N-terminals of chaperones or CTL epitopes were crucial in APC internalization or in peptide delivery to the MHC class I-related pathway. Later, Udonon *et al* reported a similar experiment but with HSC70 [131]. In this experiment the authors synthesized both N- and C-terminal HSC70 fusion proteins with several peptides without linkers. It was observed that either N- or C-bound fusion proteins were able to elicit peptide specific CTL response, suggesting that neither the N- and C-terminals of HSC70 nor the N- and C-terminal flanking linkers are essential for this process. Moreover, Udonon *et al.* synthesized several deletion mutants of HSC70 with a lack of N-terminal amino acids fused to the peptide. Such a mutant that does not bind ATP still elicited effective CTL response. Thus, it was observed that the critical region of HSC70 for re-presentation consists of 280-385 residues of the ATPase domain [131]. It was also observed that HSP-based fusion proteins were cross-presented by DCs if fused with MHC class II epitopes [132]. Such HSC70 fusion proteins with MHC class II epitopes are able to activate CD4⁺ T cells, resulting in the potential for CTL response. An increased population with much more efficient activation of CD8⁺ T cells was observed *in vivo* if HSC70 fused with both MHC class I and MHC class II epitopes was cross-presented by DCs. This is consistent with another study by Takemoto *et al.*

[133]. In this study, HSP70 was fused to both MHC class I epitope and MHC class II epitope of OVA conjugated from different sides of HSP70, one to the N-terminus and one to the C-terminus. Such fusion proteins, besides showing activation of CD8⁺ T-cell response as well as CD4⁺ T-cell response, showed higher efficacy in CTL induction compared with a mixture of two fusion proteins where MHC class I and class II epitopes were fused to HSP70 separately. Furthermore, more effective cross-presentation has been observed if MHC class II epitope is fused to the N-terminus of HSP70 rather than to the C-terminus. This suggested that cross-presentation of the involved epitopes fused to HSPs, and the regular proteins, is regulated by different proteolytic processes that are responsible for N- or C-terminal peptide cleavage, and that such cleavage takes place with different efficacy on the N- or C-terminus [134]. In addition to improvements in the HSP-peptide fusion vaccine's cross-presentation, one study reported increased cytosolic delivery of HSP70-MHC class I epitope fusion protein if conjugated with polyhistidine [135]. HSP70 fused to MHC class I OVA epitope from the C-terminal and to 25 histidines from the N-terminal showed greater ability to be delivered from endosomes to cytosol *in vitro* and *in vivo*, compared with fusion protein without polyhistidine conjugation.

Although HSP-peptide agents have been shown to be excellent activators of peptide-specific cytotoxic response in the laboratory, they have failed to show broad activity in clinical trials for autologous HSP vaccines (Table 4). As for HSP-peptide complexes and fusion proteins, clinical trials have not been undertaken. There are also no studies comparing the efficacy of HSP-based autologous vaccines with reconstituted *in vitro* complexes and fusion proteins. Thus, it cannot be precisely confirmed which type of HSP-based vaccine is preferable, since each has specific advantages and disadvantages. Nowadays, the capabilities of HSPs as adjuvants for vaccines is quite well investigated, and studies of antitumour vaccines focus more on the search for antigens or their epitopes that can provide strong antigen-specific response in association with adjuvants. To date, several studies have reported the application of several TAAs fused with or in complex with HSPs, as possible antitumour vaccines. Most of these TAAs are cancer-germline antigens due to the fact that these antigens are more investigated than others, but more tumour-associated antigens will be explored in the future as prospective anticancer vaccine components.

Table 4. Summarized data on clinical trials of HSP-based vaccines used for cancer immunotherapy according to <http://clinicaltrials.gov/>.

Title of the Study	NCT ID	Intervention (According to Trial Description)	Used HSP (According to Nomenclature (Table 1))	Conditions	Status
Autologous tumor-derived preparations					
GP96 Heat Shock Protein-Peptide Complex Vaccine in Treating Patients With Recurrent or Progressive Glioma	NCT00293423	gp96-peptide complexes	HSPC4/gp96	Brain and central nervous system tumours	Completed
Vaccine Therapy in Treating Patients With Stage I or Stage II Pancreatic Cancer	NCT00003025	Vitespen (gp96-peptide complexes)	HSPC4/gp96	Pancreatic cancer	Completed
Study of Heat Shock Protein-Peptide Complex (HSPPC-96) Versus IL-2/DTIC for Stage IV Melanoma	NCT00039000	Oncophage (HSPPC96-peptide complexes)	HSPC4/gp96	Malignant melanoma	Completed
Vaccine Therapy in Treating Patients With Recurrent Soft Tissue Sarcoma	NCT00005628	Vitespen (HSPPC96-peptide complexes)	HSPC4/gp96	Sarcoma	Completed
Trial of Heat Shock Protein Peptide Complex-96 (HSPPC-96) Vaccine	NCT02722512	HSPPC96-peptide complexes	HSPC4/gp96	Glioblastoma multiforme, grade III astrocytoma, ependymoma	Recruiting
Research for Immunotherapy of Glioblastoma With Autologous Heat Shock Protein gp96	NCT02122822	gp96-peptide complexes	HSPC4/gp96	Glioma	Ongoing
A Safety and Effectiveness Study of Vaccine Therapy in Patients With Indolent Lymphoma	NCT00081809	HSPPC96-peptide complexes	HSPC4/gp96	Lymphoma	Completed
Clinical Trial Studying a Personalized Cancer Vaccine in Patients With Non-metastatic Kidney Cancer	NCT00126178	Oncophage (HSPPC96-peptide complexes)	HSPC4/gp96	Renal cell carcinoma	Terminated
Study of the Feasibility to Derive Vaccine From Tumor Tissue in Patients With Non-Small Cell Lung Cancer	NCT00098085	HSPPC96-peptide complexes	HSPC4/gp96	Lung cancer	Completed
Study Using Vaccination With Heat Shock Protein 70 (HSP70) for the Treatment of CML in Chronic Phase	NCT00027144	HSP70-peptide complexes	HSPA1B/HSP70	Leukemia	Completed
Recombinant preparations					
Vaccine Therapy in Treating Patients With Stage III or Stage IV Melanoma	NCT00005633	Tyrosinase and gp100:209-217 peptides fused with OVA BiP peptide and HSP70	HSPA1B/HSP70	Melanoma	Completed
Vaccine Therapy in Treating Patients With Advanced Stage III-IV Melanoma	NCT01744171	HSP110-gp100 complexes	HSPH2/HSP110	Melanoma	Recruiting

An interesting view on the underlying mechanisms of anticancer vaccine effects has recently been proposed. Large numbers of activated antitumour antigen-specific T cells are already circulating in the blood during the existence of the tumour before vaccination [136]. Nevertheless, the local immunosuppressive micro-environment formed by the tumour leads to the anergy of T cells that are already present as well as preventing penetration of newly activated T cells through this milieu. This hypothesis, proposed by Coulie *et al.* in their review, is based on the observation that after vaccination, the frequency of anti-vaccine T cells in a regressing tumour in the most responsive patients can be very low; about 1 per 10^6 tumour cells [62]. During tumour regression a substantial expansion of pre-existing and new T cells specifically responding against antigens of the same tumour was also observed, and their frequency can be 10,000 times higher than anti-vaccine T cells [62]. Based on these observations, the hypothesis is that a small number of T cells penetrate the tumour milieu after vaccine activation and attack the tumour cells. Recognition of tumour antigenic epitopes by these T cells leads to their activation followed by cytokine production that focally reverses the immunosuppressive micro-environment into an immunostimulatory micro-environment. This, in turn, leads to the awakening of anergic T cells that are already present in the tumour, and also stimulates new T cells. Something like a chain reaction begins mobilizing all antitumour T cells present in the tumour milieu, while vaccine activated T cells produce the “spark” that triggers the reaction [62].

Tumour antigens themselves, even tumour-specific antigens, could encounter inefficient antigen presentation, resulting in a weak immune response. It was noted above that tumour-specific antigenic peptides fused to HSP could be presented by APCs much more efficiently than a tumour-specific antigen alone [69]. Thus, HSPs fused with or in complex with TSAs have higher immunogenic properties. Well-selected epitopes of antigenic peptides that are strictly specific for tumours and have a high affinity with the patient's MHC molecules also play a significant role. Following these observations, the cross-presentation efficacy and immunogenicity of anticancer vaccines could be increased, which, in turn, will lead to an increase in the anti-vaccine specific T-cell population, to increasing numbers of T cells capable of penetrating the tumour milieu or to increasing the quantity of “sparks” which are capable of reversing the tumour micro-environment.

CONCLUSION

To date, a number of tumour-specific HSP-based vaccines containing TSA epitopes have already shown good effects *in vitro* and in animal models. Most of them contain epitopes of cancer-germline antigens, as so far, these have been studied the most. These vaccines elicit responses against malignant cancers such as melanoma, lung carcinoma, breast carcinoma and prostate carcinoma. Moreover, other members of the TAA and TSA groups are currently being extensively studied. We are confident that novel antigens' epitopes used in HSP-based vaccines will increase efficacy, and allow for an extension of the range of target tumours. Thus, the recently reported cancer-retina antigens already offer an expanded list of cancers that could possibly be treated in this manner, such as renal cell carcinoma, gastric cancer, oesophageal carcinoma, ovarian cancer and colorectal cancer. With regard to the TSAs already discovered, more of their epitopes should be studied in order to produce a more specific and more immunogenic T-cell response.

Combining highly specific and immunogenic TSAs with HSPs as adjuvants, allows for the production of a palette of antitumour vaccines, which will be effective against a wide range of oncological disease. Due to the advantages of HSP-based vaccine production and action, such vaccines are perfect for mass administration at different cancer stages, or even for preventive administration in high-risk situations when oncogenic viral infections or aberrant protein expressions have been diagnosed. Post-operative administrations of anticancer vaccine will also be appropriate for prophylaxis of metastasis and recurrence.

LIST OF ABBREVIATIONS

ADP	=	Adenosine diphosphate
APC	=	Antigen-presenting cell
ATP	=	Adenosine triphosphate
CAR	=	Cancer-associated retinopathy
CRA	=	Cancer-retinal antigen
CTL	=	Cytotoxic lymphocyte
DC	=	Dendritic cell
DNA	=	Deoxyribonucleic acid
GnRH	=	Gonadotropin-releasing hormone
HLA	=	Human leukocyte antigen
HPV	=	Human papilloma virus

HSP = Heat shock protein
 IFN = Interferon
 IL = Interleukin
 MCP-1 = Monocyte chemoattractant protein 1
 MDSC = Myeloid-derived suppressor cell
 MHC = Major histocompatibility complex
 NK = Natural killer
 PDE6 = Phosphodiesterase 6
 PNS = Paraneoplastic syndrome
 PSCA = Prostate stem cell antigen
 TAA = Tumor-associated antigen
 TCP1 = T-complex protein 1
 TLR4 = Toll-like receptor 4
 TNF = Tumor necrosis factor
 TRP2 = Tyrosinase-related protein 2
 TSA = Tumor-specific antigen
 β -hCG = β -Human chorionic gonadotropin

CONSENT FOR PUBLICATION

Not applicable.

CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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