

REVIEW

## Feasibility Analysis of p62 (SQSTM1)—Encoding DNA Vaccine as a Novel Cancer Immunotherapy

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Cancer immunotherapy is a thriving field, but its clinical achievements are modest so far. One of its major hurdles seems to be finding a feasible cancer antigen as a target for immune response. After many years of research, three major criteria for choice of tumor antigens emerged. An antigen should be: (i) immunogenic; (ii) essential for cancers cells (to avoid its loss through immunoeediting), but dispensable for normal tissues to reduce the risk of toxicity, and (iii) overexpressed in tumors as compared to the normal tissues. Here we argue that p62 (SQSTM1), a protein involved in autophagy and signal transduction, fits all the above criteria and can be chosen as a novel cancer antigen. Accordingly, we carried out an extensive study and found antitumor and antimetastatic activity of p62-encoding DNA vaccine in five types of commonly used transplantable tumor models of mice and rats, and spontaneous tumors in several dogs. Given that toxicity of p62 vaccine was minimal, if any, we believe that p62-encoding vaccine merits further clinical development.

**Keywords:** Autophagy, cancer vaccine, transformation, tumor antigen

### INTRODUCTION

After decades of drawbacks, cancer immunotherapy finally started to deliver its promise. As far as radiation and conventional cytostatic drugs mainly achieved its pinnacle in therapeutic efficiency and there is not much space left for their improvement, oncologists see the future of the anticancer treatment mainly in two approaches: targeted therapy and immunotherapy. However, for most common human cancers such as breast and prostate cancers there were no specific targets found so far (except Her2 for a fraction of breast cancers). An emerging approach to treat such tumors is to boost tumor-specific immune response. This approach is based on a valid assumption that tumor development and growth is accompanied by suppression of host's immune system and/or escaping from immune surveillance; therefore stimulation of immunity or targeting hidden tumor antigens should lead to anti-tumor response. The BCG (Bacillus Calmette–Guérin) vaccine used to treat nonmuscle invasive form of bladder cancer is early successful example of such immune booster, which works, apparently, via stimulation of local innate immune response [1]. Currently, there are also several

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Accepted 10 August 2014.

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immunotherapeutics approved by FDA or in phase III clinical trials, such as monoclonal antibodies to PD-1 and CTLA-4 which are effective in metastatic melanoma [2]. Their activity is based on relieving immunosuppression caused by tumors, and their efficiency in clinics (although only in fraction of patients) with rather mild toxicity is a proof of principle that immune system of a cancer patient is not completely disabled and can be awakened.

Another mainstream of cancer immunotherapy is based on boosting patient's immune response to cancer-specific antigens (Ag) by vaccines. Accordingly, first were approved the vaccines against cervical cancer target Ag of human papilloma viruses (e.g., E6/E7 antigens of HPV16 and HPV18). Except for head and neck cancer where HPV is also involved [3], or hepatoma, where HCV plays a pathological role [4], a vast majority of human tumors, however, are not caused by viruses, therefore the choice of a tumor Ag is challenging. Furthermore, anticancer vaccines tested so far are not effective, in particular, since they apply selective pressure on cancer cells, which led to the loss of the vaccine-encoded Ag (immunoediting) and resulted in the tumor relapse [5]. As a result, despite numerous attempts, there is only one anticancer vaccine approved so far for common cancer (Provenge for prostate cancer), with PAP (prostate alkaline phosphatase) as an Ag [2]. However, this vaccine has rather limited effect (increase in survival by only 4 month), requires sophisticated *in vitro* production, and is very expensive. Despite these drawbacks, it demonstrates that host's immune system can be enforced to fight cancer, although with an additional help from *in vitro* propagated immune cells [6].

## DNA VACCINES

DNA vaccine is an antigen-encoding vector which is administered to the patient in order to elicit immune response. Typically, a DNA vaccine vector is a plasmid, circular double stranded bacterial DNA. DNA vaccines enter myocytes and tissue-resident APC (Ag-presenting cells, e.g., macrophages) which results in intracellular synthesis of vaccine-encoded tumor antigen. After the vaccine-encoded protein is expressed and processed, antigen-derived peptides are presented to naïve T-cells with subsequent generation of Ag-specific cytotoxic CD8<sup>+</sup> T-cells (CTL) and humoral immune response [7, 8]. The key difference between a DNA vaccine and a protein vaccine is that the antigen is expressed intracellularly. As a result, the antigen undergoes posttranslational modifications and antigen presentation through an entire array of naturally occurring intracellular mechanisms, which leads to several advantages. For example, DNA vaccines can induce very strong T-cell and B-cell responses even if amounts of antigen produced *in situ* is minimal [9]. Another benefit of DNA vaccines is modulation of protein processing rate. For example, a DNA vaccine can encode two forms of an antigen, proteosome-resistant and proteosome degradable forms. It was shown that combination of these two forms elicits stronger immune response than either of these two forms alone [10]. In the future, vaccine-encoded Ag can be modified, so it can be produced in a form with an optimal rate of proteosome degradation of an encoded protein. Furthermore, an order to modulate an intracellular Ag fate, DNA vaccines can be engineered to express a tumor Ag fused with an adjuvant protein, for example, polyglutamine sequence, inducing intracellular self-binding and aggregate formation of the vaccine-encoded antigen, which also lead to better immunogenicity [11]. Until recently, there were two significant drawbacks of DNA vaccine methodology. First, DNA vaccines could not be used for cytotoxic proteins because high level of their expression would kill the vaccine-transfected cells. Second, for some Ags, extracellular expression of their wild-type protein forms could lead to manifestation undesirable/toxic activity of the Ag protein. At the same time, it was not possible to

inactivate negative functions of the Ag protein introducing mutations into the plasmid, because a mutated gene possessed a very low expression level. Due to developments of past decade, a vaccine-encoded protein can be modified to eliminate its negative and/or dangerous properties while preserving all immunogenic domains [12]. Utilizing plasmids as a backbone provides significant benefits. Bacterial sequences such as unmethylated CpG islands in the plasmid vector operate as an adjuvant, stimulating activation of TLR9 [9]. From the point of view of public health feasibility, DNA vaccines can be generated in large amounts and with clinical grade purity in inexpensive and rapid fashion; they are safe and highly stable comparing to protein vaccines.

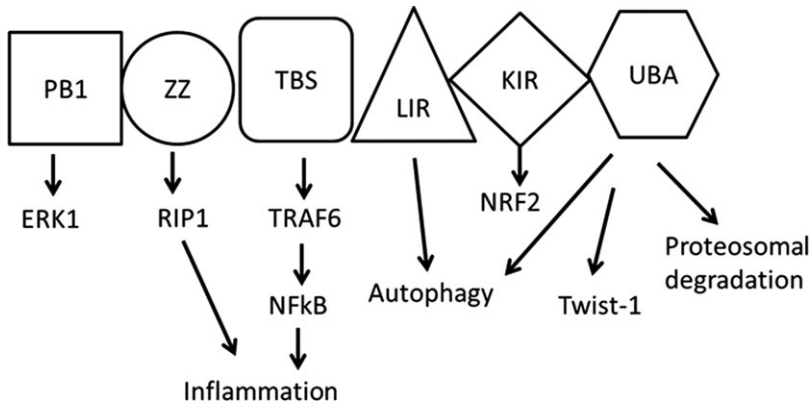
Despite their great promise DNA vaccines face two major stumbling blocks: (i) immune response they elicit may be strong enough for small animals, but not for humans (see ref. [13] for review); and (ii) mutations introduced into the antigen may alter its mRNA structure, which may lead to severe reduction in expression level [14]. Nevertheless, there are several DNA vaccines approved already for veterinary applications, including anti-melanoma DNA vaccine (Oncept) for dogs [15]. Among approaches to increase efficiency of anticancer DNA vaccine in humans are improving its administration (e.g., electroporation and gene gun which increases delivery of plasmid DNA by several times) and use of enhancers of immune response (e.g., GM-CSF, IL-2), which can be also coded by plasmid DNA [9]. Use of viral vectors such as retroviruses, lentiviruses, and adeno (or adeno-associated) viruses, although increasing delivery of DNA, but also have its limitation: possible carcinogenesis due to insertion of viruses in host genome, immunogenicity, broad tropism, limited packaging capacity, and difficulty of viral production [16]. To avoid these drawbacks of viral vectors, current development of material sciences and nanotechnology, as well as in nucleic acid chemistry led to emergence of more efficient nonviral delivery system (e.g., lipid-based and polymeric) some of which are now being tested in clinical trials (see ref. [16] for review).

According to ClinicalTrials.gov, there are 190 trials listed of cancer treatment which used or currently using DNA vaccination alone or with some combination. Among targeted cancer Ag are E6/E7 proteins of HPV-16 for cervical cancer, alpha-fetoprotein for liver cancer, CEA for colon cancer, TRP2 for melanoma, PAP for prostate cancer and some others [9].

Furthermore, there are several DNA vaccines approved already for veterinary applications, including antimelanoma DNA vaccine (Oncept) for dogs [15], demonstrating feasibility of DNA vaccination.

## **P62 AS A NEW CANCER ANTIGEN**

In 2009, a group of specialists in cancer immunotherapy made an attempt to prioritize cancer antigens for acceleration of translational research (National Cancer Institute Pilot Project) [17]. They created nine criteria and evaluated 75 tumor antigens being studied in clinics and preclinically at that time. The first ranking criterion (weighting 31%), unsurprisingly, was therapeutic function (i.e., at least some efficiency in clinical trials), then, in descending order: (2) immunogenicity; (3) role of Ag in oncogenicity; (4) specificity; (5) expression levels and percent of antigen-positive cancers; (6) stem cell expression; (7) number of patients with Ag-positive cancers; (8) number of antigenic epitopes; (9) cellular location of Ag expression; first four criteria were the most important, giving in total 79% contribution in prioritization [17]. If we exclude first therapeutic criterion to choose an Ag for preclinical studies, there will be only three major criteria left. In other words, ideally, an antigen for anticancer vaccine should be: (i) immunogenic; (ii) essential for cancers cells (to avoid its loss through immune-editing), but dispensable for normal tissues to reduce the risk of toxicity, and (iii)



**Figure 1.** Structure of p62 (SQSM1) and functions of its domains in cell signaling and protein degradation.

overexpressed in tumors as compared to the normal tissues. We hypothesized that p62 protein (sequestome 1) may be such an excellent target as a cancerAg.

p62 protein is a major player in selective macroautophagy [18] and serves as a signaling hub for several signal transduction pathways, among them NF- $\kappa$ B, TRAF6, MAP kinases, Twist1 etc. [19–22] (Figure 1) Importantly, p62 is dispensable for normal tissues, but essential for development and survival of tumors (Table 1). First, p62 knockout mice are viable and demonstrate only minor anomalies (later-onset obesity) [23], indicating that, at least under normal conditions, other proteins can substitute functions of p62 (e.g., p62 homolog NBR1 in autophagy) [24]. But as for tumor development, the situation is quite different. At least in several mouse models studied, knockout of p62 prevented or markedly delayed development of cancer caused by oncogenes (Table 1). Furthermore, fully transformed cells do not lose its dependence on p62 since its knockdown causes inhibition of growth or loss of viability (Table 1). Thus, tumors, in contrast to normal tissues, become dependent on p62. Although p62 is not oncogene per se, such dependence of tumors on some proteins is a well-known phenomenon called “nononcogene” addiction. According to this notion, proteins dispensable for normal cells become necessary for transformed cells [25]. Along with oncogenes such as *myc* and *ras* whose inactivation leads to tumor suppression or even eradication, these nononcogenic proteins are also considered as good therapeutic targets especially when oncogenes are difficult to find or they are undruggable.

**TABLE 1.** Role of p62 in human cancer.

Cancer type	High p62 expression	Correlates with progression	Depends on p62	Refs
1. Breast	+	+	ND	[39, 40]
2. Colon	+	+	ND	[41]
3. Glioblastoma	+	+	+	[42]
4. Kidney	+	ND	+	[43]
				[44]
5. Liver	+	ND	+	[45]
6. Lung	+	+	+	[26, 46]
7. Melanoma	+	+	ND	[47]
8. Myeloma	+	ND	+	[48]
9. Prostate	+	ND	ND	[49, 50, 51]
10. Pancreas	+	ND	+	[27]

ND - not determined.

Which of the p62 activities are critical for tumorigenesis? It appears that autophagy at early stages of tumor development is not required but rather detrimental. At these stages, p62 is important as a signaling hub, in particular, by activation of proinflammatory and prosurvival NF- $\kappa$ B pathway [19, 20, 26, 27] or Twist1, which is involved in cell proliferation, migration, and metastases [22] (Figure 1). In contrast, at later stages of tumor development, autophagy plays a distinct protective role, and its inhibition per se has antitumor effect. Some tumors such as pancreatic cancer become addicted to autophagy, and accordingly, there are several ongoing clinical trials with autophagy inhibitor chloroquine alone or in combination with chemotherapy [28, 29]. Whatever the mechanisms of tumor dependence on p62, this is apparently a wide-spread phenomenon (Table 1).

Another requirement for being a good Ag for anticancer vaccine is the higher expression level in tumor tissue. Indeed, according to data of OncoPrint (the largest database of human cancer microarrays) and other reports, at least 10 various types of human cancer have high levels of p62 comparing to normal tissue (Table 1). For instance, in melanoma, p62 levels are 10-times higher (OncoPrint). Furthermore, in several types of tumors, levels of p62 increase during progression (Table 1). There are several mechanisms driving p62 overexpression in cancer. We have found that common oncogenes such RAS, PIK3CA, and Her2 can induce p62 *in vitro* [30]. In addition, tumor microenvironment such as inflammation and oxidative stress can activate p62 via NF- $\kappa$ B [27] and NRF-2 [31] transcription factors. Interestingly, activation of these transcription factors also depends on p62 which leads to a positive feed-back loop [27, 31] (Figure 1).

The majority of cancer Ags targeted so far are extracellular membrane proteins, but p62 is an intracellular protein. However, recently accumulated data indicate that intracellular proteins also can be feasible Ags for antibody response, for instance survivin, PRL-3 cancer-associated phosphatase, [32–34], or WT1 [35]. There are several proposed mechanisms of targeting of these proteins by immune system: (i) uptake of antibodies (e.g., via endocytosis) and neutralization of the Ag; and (ii) displaying of the Ag on the surface via unconventional secretion pathway; [36, 37]. Relative contribution of these mechanisms in immune response to intracellular Ags currently unknown, but at least in case of PRL-3 oncoprotein, both B-cells and NK cells seems to be necessary, whereas T-cells are dispensable [38]. At the same time, antibody to WT1 was effective even in SCID mice (i.e., without both T- and B-cells) [35]. Last but not least, proteolytic fragments of intracellular p62 may be presented by MHC-I molecules to attract CTL, which makes DNA vaccines and DNA vaccination approach particularly attractive.

## ANTITUMOR EFFECT OF P62 DNA VACCINE

Based on above considerations, we have chosen p62 as an Ag for a DNA vaccine and evaluated its antitumor effect. In studies of hundreds of animals, p62 vaccine has proven its effectiveness in five kinds of solid tumors in mice and rats: lung and breast carcinomas, melanoma and sarcoma (Table 2) [30]. More importantly,

TABLE 2. Antitumor and antimetastatic effect of p62 vaccine.

Tumor	Animals	Effect on tumor	Effect on metastases
B16 melanoma	Mice	Inhibition of growth by 3-times	Suppression by 4-times
Lewis lung carcinoma	Mice	Inhibition of growth by 3-times	Suppression by 4-times
Sarcoma 37	Mice	Inhibition of growth by 4-times	Suppression by 6-times
Breast carcinoma Ca755	Mice	Increase in survival by 70%	-
Breast carcinoma T5	Rats	Increase in survival by 50%	-

TABLE 3. Preclinical studies for the p62 vaccine safety.

Study	Animals/doses	Conclusion
Acute i.m. and i.p. toxicity	Rats, mice, guinea pigs 1, 5, 10, 50 ETD	No acute toxicity observed
Chronic toxicity upon i.m. daily administration for 90 days	Rats - 1,5,10, 50 ETD	Low hazard
Allergic activity	Dogs - 1, 10 ETD Guinea pigs, 1, 10 ETD	Low toxicity No anaphylactic shock or local allergic reaction
Immunological safety	Mice, 1,10 ETD, 5 times i.m	No effect on B- and T-cell response
Embryotoxicity and teratogenicity	Rats, 1, 10 ETD, 5-times i.m.	No embryotoxicity or teratogenicity

ETD—effective therapeutic dose.

it also possessed strong antimetastatic activity in three models of metastases: spontaneous metastases to lung (Lewis lung carcinoma), to regional lymph nodes (sarcoma 37), and induced metastases (by i.v. infection) in B16 melanoma. We also found that, at least in case of lung carcinoma and melanoma, p62 vaccine decreased both the number and size of metastasis, indicating that it suppresses both colonization of lung by tumor cells (e.g., formation of micrometastases), as well as growth of established metastases. Of note, in case of melanoma, p62 vaccine was effective not only in preventive (given 2 weeks before tumor inoculation), but in therapeutic setting as well (after tumor inoculation), suppressing both primary tumor and metastases [30]. This metastatic melanoma model, where tumor cells are already in circulation (after i.v. injection) and p62 being injected afterwards more closely resembles human cancer, where many patients are first diagnosed with cancer when metastatic process is already underway. Since great majority of patients (about 90%) die from metastases rather than from primary tumors, such antimetastatic effect of p62 vaccine seems very encouraging.

Also encouraging are our preliminary data about the effect of the vaccine on spontaneous tumors in dogs, in particular, mammary tumors. Given on compassionate use basis to several dogs with incurable cancers, p62 vaccine halted the progression of the disease and markedly improved animal's well-being (manuscript in preparation).

As a next step in preclinical development of p62 DNA vaccine as a candidate for future clinical trials, its toxicological properties were tested in mice, rats, guinea pigs, and dogs. As one can see from Table 3, the vaccine was well-tolerated, without acute or chronic toxicity, allergic activity, and it did not cause embryonic toxicity and teratogenicity. There was no hematotoxic, hepatotoxic, and nephrotoxic effects, as well as effects on carbohydrate and lipid metabolism. Furthermore, histological examination of brain, heart, lung, liver, kidney, spleen, thyroid, and thymus did not find any anomalies (Shifrin *et al.*, unpublished data). Therefore, antitumor and anti-metastatic activity of p62 vaccine was not accompanied by any significant side effects, as expected for DNA vaccines.

Overall, preclinical data presented above justifies further veterinary research and human clinical testing of p62-encoding DNA vaccine as a novel anticancer agent.

## FUNDING

The research covered by review was partially supported by CureLab Oncology Inc.

## Declaration of Interests

The authors are employees of CureLab Oncology Inc. The authors alone are responsible for the content and writing of the article.

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