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TITLE

Method of Detecting and Targeting Mutations in Cancer

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Abstract

While there are many differences between tumor and non-tumor cells, the basic underlying distinction is in the DNA. Tumor cells harbor mutations, at least some of which are not present in non-tumor cells. Thus, a method of directly targeting cells containing specific mutations has potential for detection or treatment of cancer without the toxicity associated with more indirect approaches. Also, as mutations are a necessary component of malignancy, such a method is potentially applicable to all tumors.

I propose a method by which several recently developed techniques can be utilized in a novel way to accomplish the goal of directly targeting mutations for cancer detection and therapy. The model can be summarized as follows: (1) Determine potential target mutations present in tumor cells but not non-tumor cells. (2) Construct molecules that will bind to DNA at the sites of mutation, but will not bind to DNA in normal cells. And, as a consequence of the molecules binding to the mutation, the cells will be destroyed. (3) Deliver these molecules to all cells (or at least all tumor cells). I hypothesize that such molecules can now be constructed using sequence-specific DNA binding proteins (such as customized zinc-finger DNA binding proteins) fused to transcriptional activator domains (such as VP16) and reporter or toxin genes. The necessary genes can be linked to the DNA binding proteins utilizing a recently described method based on expressed protein ligation.

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Introduction

Most, if not all, tumors are thought to arise due to the accumulation of mutations, providing the cells with the necessary characteristics for malignancy. Yet, this most fundamental attribute of cancer has not been amenable to targeting for therapeutic or *in vivo* diagnostic purposes. The most obvious factor limiting mutations as potential targets is their concentration—there may be only a single copy of a specific mutation per cell. Thus, in order to have a useful effect any approach that targets DNA must incorporate significant amplification.

In recent work, the ability to construct zinc-finger (ZF) DNA binding proteins designed to bind to specific, user-defined DNA sequences has been demonstrated [1-4]. These proteins have been fused to various other protein moieties, producing for example artificial transcription factors and site-specific nucleases [5-11]. Additionally, ZF proteins fused to reporter proteins have been used to detect specific DNA sequences both *in vitro* [12-14] and in plants and mammalian cell culture [15]. A limitation of these techniques is the lack of amplification: As there is only a single copy of reporter protein (e.g. green fluorescent protein) per target DNA site, the methods must be used either *in vitro* (with many copies of the target DNA) or targeted to highly repetitive DNA sequences.

Recently a conceptual paper was published [16] describing a technique for targeting DNA in non-tumor cells. However, key components of that method (such as split nucleases) do not currently exist so that the approach cannot be implemented at this time,

and DNA methylation and compaction in non-tumor cells may limit the method's applicability should it become possible to test it. The strategy proposed here, utilizing a different method and with a different target, does not have these limitations.

Hypothesis

In this article I propose a new strategy, with potential to directly target mutations *in vivo*. I hypothesize that DNA binding proteins, constructed to bind to mutations, can be joined to other moieties as described below, with the resulting molecules enabling detection and treatment of tumor cells based on these molecular differences (i.e. mutations) from non-tumor cells.

The method is illustrated in Figure 1. Two DNA binding proteins (assumed to be ZF proteins) are constructed, designed to bind near to one another on the cellular DNA. At least one must bind at a mutation not present in normal cells; however, the other may bind to non-mutated DNA as both proteins must be bound for the mechanism to produce an effect. Attached to one ZF protein (which can be constructed as a fusion protein via standard techniques) is a transcriptional activator (e.g. VP16). Attached to the other is DNA with an appropriate promoter followed by DNA to be transcribed (typically a reporter gene for tumor detection, or a toxin gene for therapy).

If the two ZF proteins are bound near one another, the transcriptional activator will interact with the promoter, resulting in markedly increased transcription of the attached gene. If the proteins are not attached near one another (Figure 2) there will be some

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occasional interaction and baseline transcription, but at a far reduced level. Thus, there will be a much larger amount of the desired protein in cells with target mutations.

The method employs techniques which have previously been demonstrated to work *in vitro* and/or in mammalian cell culture (custom zinc-finger DNA binding proteins and artificial transcription factors, regiospecific protein-DNA conjugate synthesis [17-26], and elements employed in one- and two-hybrid systems [27-29]), but now combined and modified in a way that allows targeting of native, cellular DNA. This is, to my knowledge, the first proposal of fusing reporter or toxin gene DNA (as opposed to single proteins) to ZF DNA binding proteins, enabling significant amplification of the gene product for the purpose of directly targeting mutations in tumor cells.

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Evaluation of the Hypothesis

The principle of having a transcriptional activator held near a promoter has been successfully demonstrated in one- and two-hybrid systems, in the evaluation of DNA binding proteins and protein-protein interactions (Figure 3). Such systems are not useful for detecting cellular DNA, however, because in cells there will not generally be an appropriate promoter and reporter or toxin gene immediately adjacent to the site of mutation. Therefore one- and two-hybrid systems introduce a reporter plasmid with a DNA protein binding site adjacent to the necessary promoter and reporter gene. Adapting this approach to detect cellular DNA requires a method of bringing the necessary foreign DNA (promoter plus reporter or toxin gene) to the vicinity of the mutation so that the bound transcriptional activator can interact with it.

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While the foreign DNA may be attached to the ZF protein in numerous ways, ideally it will be done in a regiospecific manner. Non-specific attachment of the DNA (for example to lysine residues in the ZF protein) may interfere with ZF binding and thus reduce the sensitivity of the system. Various techniques have been described for regiospecific protein-DNA binding. The method based on "expressed protein ligation" [23-27] has the advantages of being regiospecific, well-tested, and simple. Also, it does not introduce large intermediate proteins which could cause problems with steric hindrance.

In order to maximize the interaction of the transcriptional activator with the promoter, appropriate linkers connecting the ZF proteins to their conjugates must be included. This is an area with much potential room for optimization, and may differ based on the separation of the two DNA binding sites. However, in general flexible linkers consisting of serine, glycine, and threonine have been shown to work well and are an appropriate starting point for investigation [30,31].

For use in eukaryotic cells, a nuclear localization signal [32] may be included in the proteins, as has been previously employed with other ZF fusion proteins [15]. In order to protect the free end of the linear DNA from exonuclease degradation, it may be helpful to form a covalently closed end using oligonucleotides ("dumbbell DNA") [33], or to otherwise modify the free end by attachment to a peptide or modification of the nucleotides at this end. Additional DNA elements, such as polyadenylation signals, will also likely be helpful in eukaryotic cells.

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Functioning of the system will require delivery of the two constituent molecules to all or most tumor cells. However, a tumor-specific delivery system is not required as the system is designed to only have an appreciable impact on tumor cells. It is expected that the molecules will be constructed *ex vivo* and delivered as protein (and protein-DNA conjugate) therapeutic agents. There is much active research into the *in vivo* delivery of such agents, including cell-penetrating peptides, liposomes, and viral vectors [34-36]. Whether or not these or other delivery mechanisms will function with this system is perhaps the largest question regarding *in vivo* implementation. However, given the general interest in delivery of protein therapeutic agents, there is likely to be significant progress in this area and testing of this approach in cell culture need not wait for the *in vivo* delivery issue to be resolved.

Other issues that may affect protein therapeutic agents generally, such as immunogenicity and toxicity, will also need to be addressed.

The genetic instability of tumors must also be considered. Research has shown that tumor cells may contain 10,000 to 100,000 mutations, although there is significant variation in genetic instability in different tumors [37]. While this may appear to pose difficulty for an approach targeting specific mutations, it is unlikely to cause significant problems in practice. The large number of mutations (many of which appear very early in tumor development) create a wealth of potential targets. While there are many mutations, the target sites for this approach are expected to be approximately 12 base pairs long (expected to be sufficient to specify a unique site in the genome), and sequencing of short segments of DNA in individual tumor cells from the same individual suggests that it is uncommon for such a short segment of DNA to be a site of repeated mutations [38]. Thus, while it will likely be necessary to target multiple mutations (particularly in

tumors with a high degree of genetic instability), the number of required sites is not likely to be large. More research on the degree of genetic instability in tumors will help to clarify this point.

Discussion

The mutations which are the root of malignancy are an interesting but difficult potential therapeutic target. The fact that there may be only a single copy of a mutation per cell poses significant challenges in terms of producing a useful signal or effect. In this article I have proposed a method which has the potential to target individual mutations, either for detection or therapy (or both—for example, HSV-tk in conjunction with radiolabeled ganciclovir or penciclovir analogs can function as both reporter and toxin [39]). It incorporates several methods which have previously been successfully implemented in mammalian cell culture, so that it is reasonable to expect that this combination will also work in culture. Use of the system *in vivo* poses further challenges, however.

In addition to therapy, the method is easily adapted to tumor detection as well, simply by employing a reporter gene (e.g. green fluorescent protein, HSV-tk, luciferase [40]) rather than a toxin gene. Also, it may be of interest to determine the prevalence or distribution of mutations (or other specific DNA sequences) in non-malignant cells. This may also be accomplished with this method.

Furthermore, any DNA difference between two cell populations may be exploited. Thus, in addition to malignancy the method has potential in treatment of infections in which foreign DNA is present within cells. Retroviral infections, such as HIV, in which the viral genome is integrated into the cellular genome, are obvious candidates.

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In summary, I have proposed a technique for directly targeting mutations as a method of detecting or treating malignancy. Experiments to test this hypothesis are currently under way.

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FIGURE LEGENDS

Figure 1: THE METHOD IN TARGET CELLS. Two zinc-finger proteins (ZF1, ZF2) are bound to the cellular DNA at nearby locations. As a result, the transcriptional activator (e.g. VP16) is held in proximity to an appropriate promoter on foreign DNA which is attached to ZF2. Following the promoter, there is a reporter or toxin gene. Interaction of VP16 and the promoter leads to transcription of the gene and production of the resulting protein.

Figure 2: THE METHOD IN NON-TARGET CELLS. In this case the target sequences for the zinc-finger proteins (ZF1, ZF2) are not present, so ZF1 and ZF2 are not generally bound near one another. As a result VP16 is not held in proximity to the promoter on the DNA attached to ZF2, and there will be less transcription and resulting protein than in target cells.

Figure 3: ONE-HYBRID SYSTEM. In a one-hybrid system a reporter plasmid is introduced into cells, along with a separate plasmid which codes for a fusion protein consisting of a DNA binding protein linked to a transcriptional activator. The reporter plasmid contains a potential site for binding of the DNA binding protein, and downstream of this is an appropriate promoter and reporter gene. If the DNA binding protein binds to the site on the reporter plasmid, transcription is activated and reporter gene product is

increased over cases where the DNA binding protein does not bind to the reporter plasmid.

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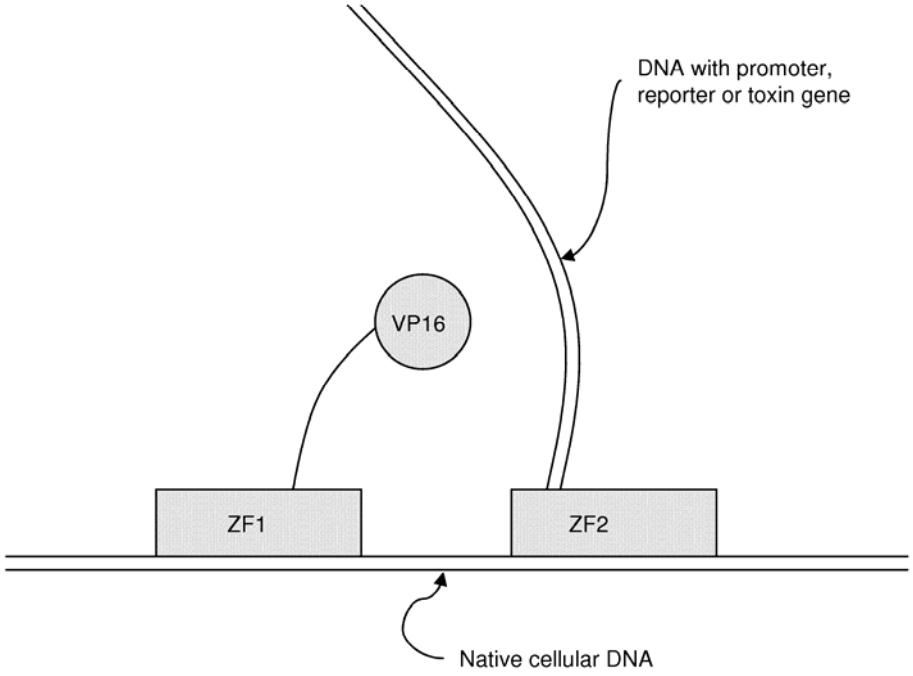

FIGURE 1

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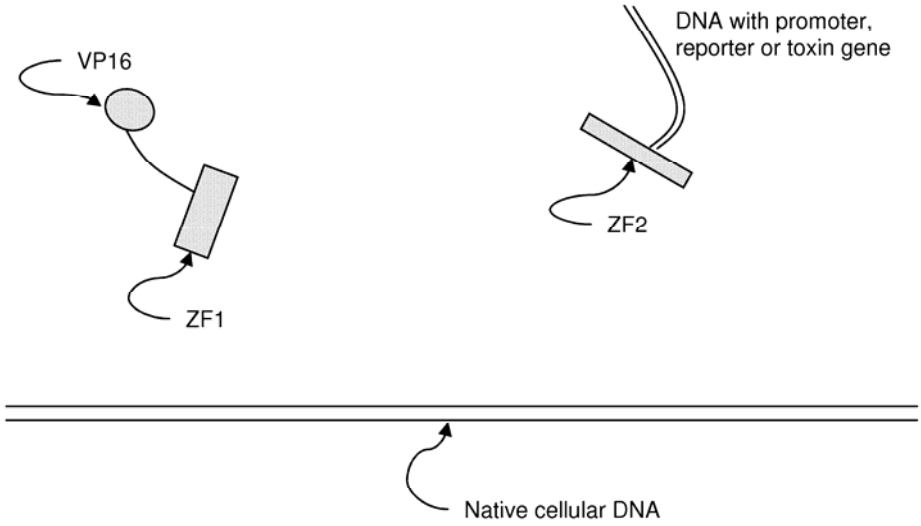

FIGURE 2

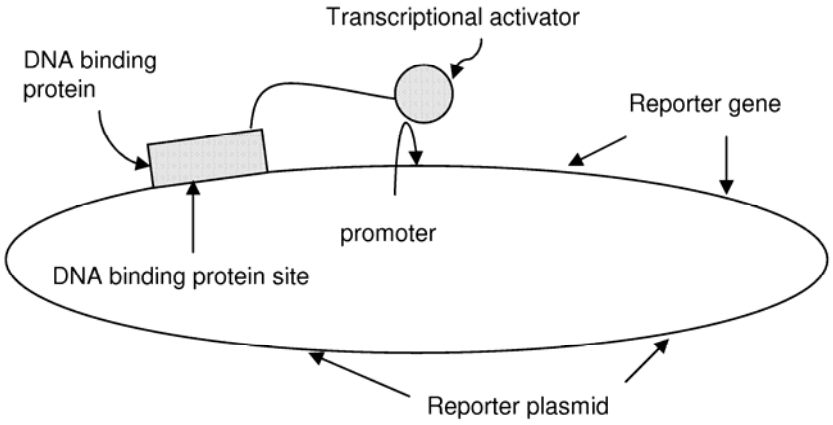

FIGURE 3

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