行政院國家科學委員會專題研究計畫成果報告

以可誘導基因功能剔除系統研究 *MYH* 基因在腫瘤化過程所扮演的角色

Study of *MYH* gene in tumorigenesis with an inducible functional knockout system

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摘　要

細胞內的氧化逆境會導致 DNA **的破壞，而這一類的** DNA **損壞是造成突變的重要原因之一。在癌細胞中，**DNA **氧化損壞濃度比相對應的正常細胞要高，顯示在腫瘤化過程中，氧化損壞未被有效率地修補而累積，因此** DNA **的氧化損壞及修補與腫瘤化過程有關。關於** DNA **氧化損壞雖然已有幾篇報告，但對於其與腫瘤形成的關係並無深入之探討，迄待努力。**

腫瘤的形成是經由多次的遺傳物質改變而來，具有較高突變潛力的細胞就有較高的可能性會轉變為癌細胞。在各種 DNA **氧化損壞中，**7,8-dihydro-8-oxo-guanine (8-oxoG) **最具有突變的潛力，它可以和胞嘧啶**(cytosine)**正常配對，也可以大約相同的親和力和腺嘌呤**(adenine)**形成錯誤配對，這種錯誤配對如果未在** DNA **複製前修補就會造成** C/G **到** A/T **的突變。**MutY(**在人類細胞是** hMYH)**就是專門修補這種錯誤配對的酵素，它會認識** A/8-oxoG **的錯誤配對，將其中的腺嘌呤切除，細胞中如果缺乏這種活性，突變率會提高數百倍。這種反突變的特性顯示** *hMYH* **可能是一個抑癌基因，但相關之研究仍付之闕如。本計畫的目地即在探討此基因在細胞腫瘤化過程中所扮演的角色。我們計畫以老鼠** NIH3T3 **為模式細胞，將老鼠的** *mMYH* **基因轉殖到** Tet-Off **系統中，使其基因表現受四環黴素**(tetracycline)**的調控，在不同濃度的四環黴素誘導下會表現不同量的** antisense RNA**，希望能藉此剔除** mMYH **的功能，再進一步利用這種可誘導式基因功能剔除細胞來研究** *MYH* **基因在氧化逆境中之功能及其在腫瘤形成與進一步癌化所扮演的角色。我們已順利將** *mMYH***基因選殖，目前正在進一步鑑定其特性。然而以人類的** *hMYH* antisense RNA **實驗，無法抑制其蛋白之表現，因此我們將改變方向，嘗試以可誘導式基因表現系統大量表現** *mMYH* **來檢查此基因表現過量對細胞的影響。**

關鍵詞：DNA **修補；**DNA **氧化損壞；可誘導式基因功能剔除系統；** *MYH*

ABSTRACT

Oxidative stress induces DNA base lesions in the cells. Elevated concentrations of these lesions were found in cancers, including invasive ductal carcinoma of the female breast and lung cancer suggesting modification in the DNA bases is a cause of mutagenesis and carcinogenesis. Although a number of DNA base lesions has been documented in the biological systems, no insight has been provided regarding relationships between these lesions and etiology of cancers.

Development of cancer is mediated through multistep genetic changes. Cells with higher mutagenic potential would be more likely to develop cancers. Among the oxidative DNA base lesions, 7,8-dihydro-8-oxo-guanine (8-oxoG) is the most common

and mutagenic one. It can base pair with either a cytosine or an adenine with equal affinity and in the latter case can cause mutation through DNA replication. The MutY (hMYH for human) protein can recognize A/8-oxoG mismatches and remove the mispaired adenine. Defect in *mutY* gene induces several hundred-fold higher mutation rates for C/G to A/T transversion. All these characteristics make the *hMYH* gene a putative tumor suppressor gene. However, no study about the possible linkage between this gene and any type of cancers was documented. Here, we propose to establish an inducible functional knockout system using Tet-Off to study the role of *MYH* gene in the tumorigenesis process. We planed to establish stable NIH3T3 clones that can express the antisense sequence of *MYH* upon tetracycline induction. Tumorigenicity potentials of these clones in the presence or absence of oxidative stress will be compared to elucidate the effect of this gene function in tumor formation and progression. During the past year, we have successfully cloned the mouse *mMYH* gene and expressed the protein in *E. coli*. We are currently further characterizing this protein. We also tried the antisense block by using human *hMYH* antisense clone. However, expression of the full-length antisense *hMYH* can not block the expression of *hMYH*. We now switch to check if overexpression of *mMYH* would alter the mutagenic property of NIH3T3 cell and increase tumorigenicity.

INTRODUCTION

Reactive oxygen species have been implicated both in the aging process and in degenerative diseases, including arthritis and cancers (Farr and Kogoma, 1991; Pacifici and Davies, 1991). Oxidative damage to DNA is one of the most important causes of spontaneous mutations and may play a role in aging and related diseases in human. The damage results from the attack of purine and pyrimidine rings by free radicals and reactive oxygen species formed as byproducts of normal cell metabolism or during oxidative stress. Among different oxidative lesions, 7,8-dihydro-8-oxo-guanine (8-oxoG) is one of the most stable products (Dizdaroglu, 1985). Ionization radiation and other treatments that generate active oxygen species also produce 8-oxoG. Besides these exogenous treatments, endogenous processes, such as electron transport or lipid peroxidation, can produce the active oxygen species that leads to 8-oxoG formation in the cells. It forms stable base pairing with a cytosine or an adenine (Kouchakdjian *et al.*, 1991; Oda *et al.*, 1991).

In *E. coli*, 8-oxoG lesion is corrected by a system involved three gene functions: *mutT*, *fpg* (*mutM*), and *mutY* (Michaels and Miller, 1992). The *mutT* gene encodes a dGTPase that can hydrolyze 8-oxo-dGTP to 8-oxo-dGMP, thus eliminating 8-oxo-dGTP from the nucleotide pool (Akiyama *et al.*, 1987; Bhatnagar *et al.*, 1991; Maki and

Sekiguchi, 1992). The C/8-oxoG pairs can be recognized by Fpg (or MutM) protein. The Fpg (MutM) has glycosylase and AP lyase activities that remove the 8-oxoG from the C/8-oxoG pairs (Tchou *et al.*, 1991). However, it can not act on A/8-oxoG mispairs. If the 8-oxoG lesion was not removed by Fpg (MutM) protein before replication, misincorporation of dA opposite the 8-oxoG lesion might occur. In this situation, MutY can remove the misincorporated adenines from A/8-oxoG mispairs and gives the DNA polymerase a chance to insert cytosine opposite to the 8-oxoG. The 8-oxoG in C/oxoG pairs is then corrected by Fpg (MutM) protein. Therefore, With the cooperative functions of MutY, Fpg and MutT proteins, *E. coli* can prevent the genotoxic effect of 8-oxoG (Fig. 1). Defects in *mutY* or *fpg* genes induce ten- to several hundred-fold higher mutation rates for C/G to A/T transversion in *E. coli*. *mutT* is also a mutator gene that increases A/T to C/G transversion rate several hundred folds higher. These characteristics make these genes putative tumor suppressor genes. However, correlation between mutation in these genes and any type of cancers is not clear.

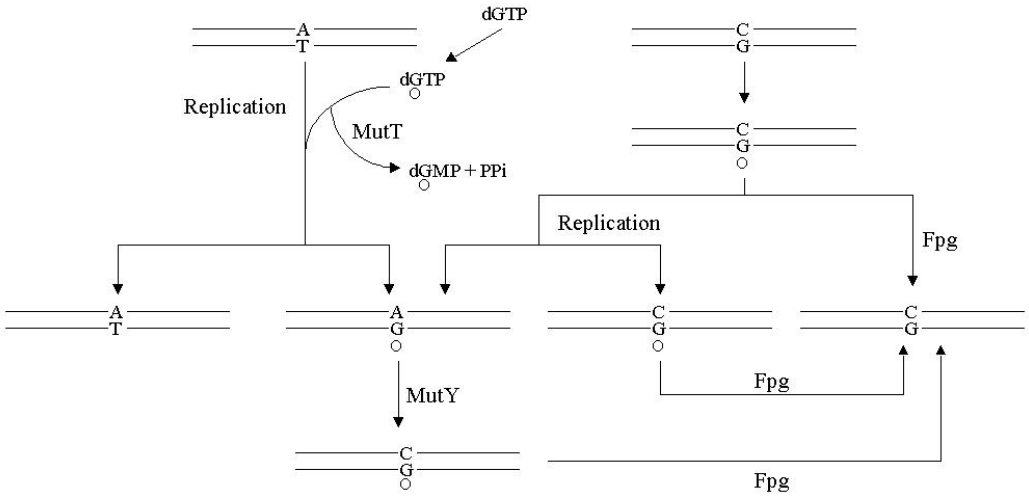

Fig. 1. The *mutY-fpg-mutT* repair system of *E. coli*.

The *mutY-fpg-mutT* repair system is conserved throughout prokaryotes and eukaryotes. It has been reported that DNA of several cancer tissues, including breast, lung, brain, and ovary, contain higher 8-oxoG concentration than that of normal tissues (Malins and Haimanot, 1991; Malins *et al.*, 1993; Olinski *et al.*, 1992). Since 8-oxoG is frequently formed in cells and is a mutagenic agent, it is important to understand how cells prevent 8-oxoG-induced mutations and how these genes might involve in cancer development. The goal of this project is to investigate the role of *MYH* in tumorigenesis. We use an inducible functional knockout system in NIH3T3 cell to study whether there

is direct involvement of this gene in tumorigenesis of cells experiencing oxidative stress. We also tried to overexpress this gene to see the effect of imbalanced expression of specific repair gene in tumorigenesis. The information resulted from this study should prompt further understanding of pathogenesis of cancers.

MATERIALS & METHODS

Cloning of the Mouse *MYH* Homologue (*mMYH*)

There are three mouse EST clones in the GeneBank that match *hMYH* sequence. Among these three EST clones, ATCC1364050R has covered almost the whole gene except the N-terminus. We use the sequence information of these clones to design primers for reverse transcriptase-based polymerase chain reactions (RT-PCR). In addition, the N-terminal sequence was cloned by 5'-RACE (rapid amplification of cDNA ends, Life Technologies) and was ligated with the EST clone to generate the full-length cDNA.

Plasmid Construction and Transfection

The *mMYH* cDNA was cloned into the tetracycline-responsive plasmid pTRE (Clontech) in the way that expression is regulated by tetracycline or its derivatives. In addition, we cloned the hygromycin-resistance gene (selection marker) into the pTRE for selection.

NIH3T3 is a transformed yet non-tumorigenic cell line and is suitable for this study. We transfected pTet-Off, the plasmid containing tTA under the control of strong CMV promoter and the neomycin gene for selection, and select stable clones. The recombinant plasmid containing *mMYH* and hygromycin genes was then transfected into the pTet-Off cells and was again selected for stable clones. Using this two-step transfection procedure, we can establish cell lines that express sense or antisense sequences in response to various concentrations of tetracycline.

RESULTS

The N-terminal fragment of *mMYH* was cloned by 5' RACE and was ligated to the EST clone ATCC1364050R to get the full-length gene (Fig. 2). The gene was cloned into pTRE plasmid at sense or antisense orientations for controlled gene expression. NIH3T3 cell was transfected with pTet-Off plasmid and the stable clone with highest expression of tTA regulator protein (as judged by β–galactosidase activity of the pTRE-derived plasmid expressing *E. coli LacZ* gene) was selected. The sense or antisense orientations of *mMYH* in pTRE were then transfected into the Tet-Off stable NIH3T3 clones and selected for hygromycin-resistant clones. More than 50 antisense clones were screened for the expression of mMYH proteins by Western blotting using anti-hMYH

antiserum. All clones expressed *mMYH* and there was no difference with or with tetracycline. Same situation happened in breast epithelial cell MCF-10A stable lines with antisense *hMYH*. The clones containing sense orientation of *mMYH* were unstable and the gene were easily lost after several passages although tetracycline was added in the culture medium to suppress the gene expression. It indicates that the amount of mMYH protein in the cell is tightly controlled. The minor overexpression of the gene from the leaky suppression of the plasmid could damage the cells. The effect of overexpression is currently under investigation.

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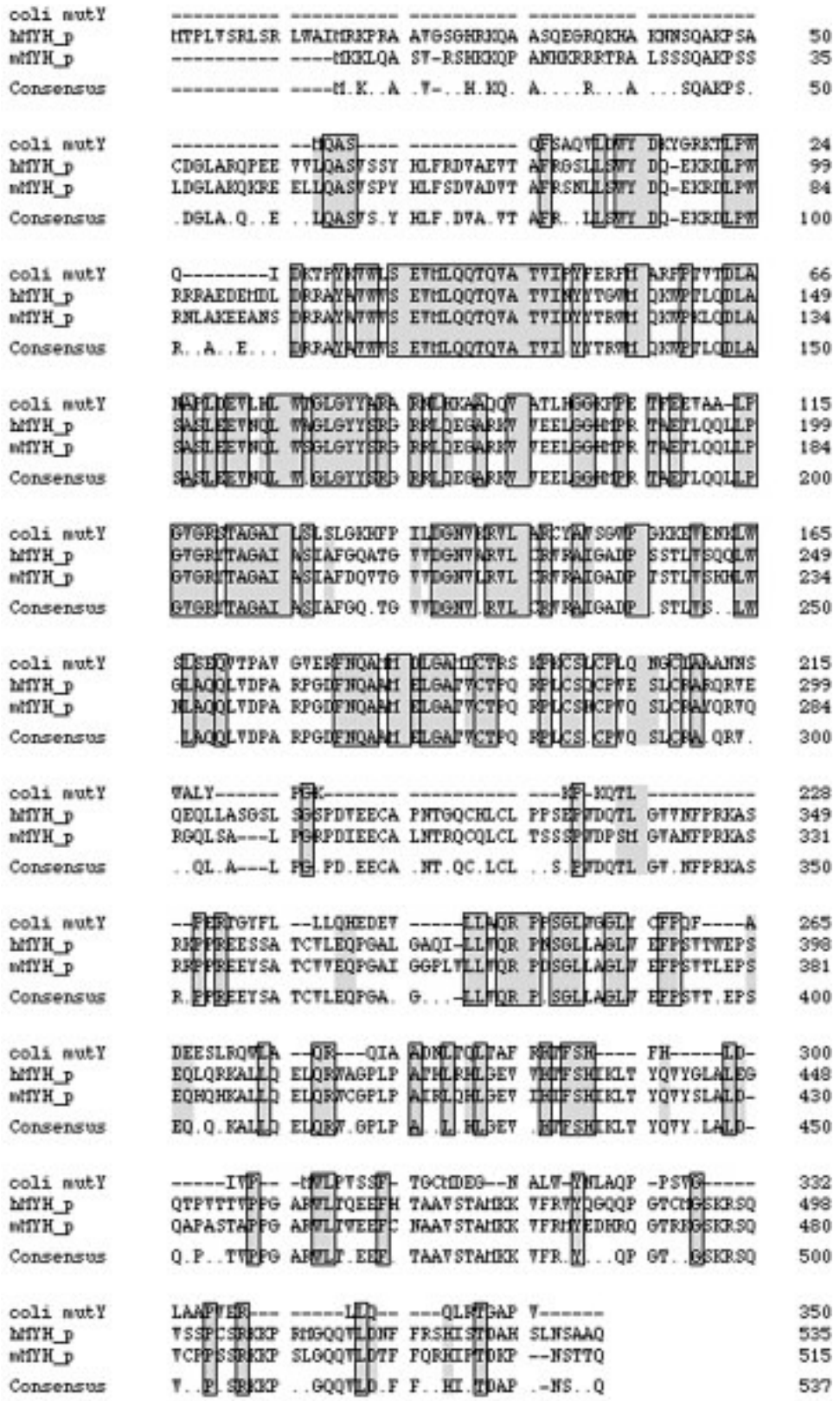

Fig. 2. Sequence alignment of *E. coli* MutY, human hMYH and mouse mMYH proteins.