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Review

Deciphering the molecular genetic basis of NPC through functional approaches

Hong Lok Lung[a], Arthur Kwok Leung Cheung[a], Josephine Mun Yee Ko[a], Yue Cheng[a], Eric J. Stanbridge[b], Maria Li Lung[a,*]

[a] Department of Clinical Oncology and Center for Cancer Research, University of Hong Kong, Pokfulam, Hong Kong (SAR). People's Republic of China
[b] Department of Microbiology and Molecular Genetics, University of California, Irvine, CA 92697, USA

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ABSTRACT

The identification of cancer genes in sporadic cancers has been recognized as a major challenge in the field. It is clear that deletion mapping, genomic sequencing, comparative genomic hybridization, or global gene expression profiling alone would not have easily identified candidate tumor suppressor genes (TSGs) from the huge array of lost regions or genes observed in nasopharyngeal carcinoma (NPC). In addition, the epigenetically silenced genes would not have been recognized by the mapping of deleted regions. In this review, we describe how functional approaches using monochromosome transfer may be used to circumvent the above problems and identify TSGs in NPC. A few examples of selected NPC TSGs and their functional roles are reviewed. They regulate a variety of gene functions including cell growth and proliferation, adhesion, migration, invasion, epithelial-mesenchymal transition, metastasis, and angiogenesis. These studies show the advantages of using functional approaches for identification of TSGs.

1. Historical perspectives

Early somatic cell fusion studies of malignant and nonmalignant mouse cells by Harris et al. [1] provided the first evidence for a class of negatively acting tumor suppressor genes (TSGs) harbored on normal chromosomes that in contrast to dominant-acting oncogenes, play a critical role in suppressing tumor formation. These initial somatic cell hybrids were non-tumorigenic. However, upon further passage tumorigenic segregants arose, which simultaneously showed concomitant loss of specific normal chromosomes [2]. These early studies were complicated by the chromosomal instability of the intraspecific mouse cell hybrids, resulting in rapid appearance of tumorigenic segregants. Confirmation of the genetic basis of tumor suppression came from studies with the chromosomally stable intraspecific human cell hybrids [3]. It was also shown that the transformed and tumorigenic phenotypes were under separate genetic control [4], again a confirmation of the Harris studies [1]. Later studies attributed this re-emergence of tumorigenicity to the subsequent loss of normal chromosomal regions, that we now know harbored TSGs, which were either defective or silenced by other means in the original tumorigenic parental cells [2]. A further refinement of the somatic cell fusion technique was developed whereby Stanbridge and others [3,4] engineered a panel of donor microcell hybrids (MCHs) containing neomycin-resistance tagged single human chromosomes originating from "normal" fibroblasts into a mouse cell background for subsequent monochromosome transfer of the individual copy of the normal human chromosome of choice into the relevant human cancer cell. With these chromosome donor cell lines, selective transfer of individual chromosomes of interest could be studied. Many investigators have used this functional approach to identify critical chromosomal regions harboring TSGs [5–14]. Although the technique of monochromosome transfer is laborious, its advantage is that the transferred normal copy of the TSG is under the control of endogenous regulatory elements, which assures more physiological expression levels of the genes of interest. Utilizing this functional rather than molecular or cytogenetic approaches, investigators in several laboratories have successfully complemented genetic defects present in the human cancer cells and used various strategies to narrow down and identify candidate TSGs associated with a number of human cancers such as those in the brain, breast, cervix, esophagus, lung, nasopharynx, ovary, prostate, and skin [5–14]. Genes identified by this functional approach were related to cell growth, immortalization, angiogenesis, invasion, and metastasis. In nasopharyngeal

Abbreviations: TSG, tumor suppressor gene; NPC, nasopharyngeal carcinoma; MCH, microcell hybrid; MMCT, microcell-mediated chromosome transfer; YAC, yeast artificial chromosome; P1, bacteriophage P1 cloning system; PAC, P1-derived artificial chromosome; BAC, bacterial artificial chromosome; TS, tumor segregant; EMT, epithelial-mesenchymal transition; LOH, loss of heterozygosity; ESCC, esophageal squamous cell carcinoma; ADAMTS9, A Disintegrin-like And Metalloprotease with ThromboSpondin type 1 motif 9; HUVEC, Human Umbilical Vein Endothelial Cell; TSP, thrombospondin; PTPRG, Protein Tyrosine Phosphatase Receptor type G; FNIII, fibronectin III; NP, nasopharyngeal; FBLN2, FiBuLiN-2; TSLC1, Tumor Suppressor in Lung Cancer 1; CADM1, Cell ADhesion Molecule 1; XMMCT, γ-irradiation microcell-mediated chromosome transfer; CRYAB, Alpha B-CRYstallin; MIPOL1, Mirror-Image POLydactylyl 1; CRIP2, Cysteine-Rich Intestine Protein 2.

* Corresponding author. Tel.: +852 28199783; fax: +852 28195872.
E-mail address: mlilung@hku.hk (M.L. Lung).

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carcinoma (NPC), using this monochromosome transfer approach, a number of tumor-related genes was identified.

2. Methodology

Functional approaches for identification of TSGs in cancer involve basically three steps [10]. The first step is the transfer of the normal DNA or cDNA into recipient cells. This is accomplished via methods ranging from whole cell fusion, microcell-mediated chromosome transfer (MMCT), spheroplast fusion, and homologous recombination of YAC (yeast artificial chromosome) to lipofection of P1 (bacteriophage P1 cloning system), PAC (P1-derived artificial chromosome), BAC (bacterial artificial chromosome), and plasmid. The major advantages of MMCT over YAC/P1/PAC/BAC transfer and lipofection are the strict control that results in transfer of only a single copy of the chromosome of interest into the recipient cells and freedom from any resultant position effects. MMCT is an ideal method for studying gene expression, as the target genes are under the control of endogenous promoters, closely mimicking the physiological environment. Therefore, non-specific effects caused by cytotoxicity or cytostatic effects that may arise from conventional transfection due to over-expression of the genes of interest can be avoided. The second step investigates the suppression of the malignant phenotype in the recipient cells of interest. The functional effects of these genes with tumor suppressor activities can be categorized depending on the type of end-point assay chosen. To identify TSGs crucial in NPC development, a functional complementation approach permitting direct suppression of tumorigenicity in nude mice was used for successful identification of TSGs involved in different biological processes that hallmark cancer development. After a long period of selection in the nude mice (>3 months), if tumors with significant sizes form, they are removed aseptically and disaggregated. The cells are then established into the tumor segregant (TS) cell lines as revertants and are used in further analysis with their matched MCHs. The third step involves analyzing the TSs in order to narrow down the critical regions (CRs) or to screen for the candidate genes associated with tumor suppression. The TSs are believed to arise due to elimination of CRs associated with tumor suppression. Loss of donor genetic materials is detected by microsatellite typing and BAC fluorescence *in situ* hybridization (FISH). In addition, the differential gene expression profiling between the tumor-suppressive MCH cell lines *versus* their matched TSs and the highly tumorigenic recipient cell line is revealed by oligo- or NotI methylation microarray analyses. Once a candidate gene is identified, the gene of interest can be subjected to the *in vivo* tumorigenicity assay using a tetracycline-regulated system for studying gene functions [15]. Fig. 1 summarizes this three-step functional approach for identifying candidate TSGs.

3. MMCT studies in NPC identify critical regions and candidate tumor suppressor genes

Using the MMCT functional approach, selected chromosomes showing high allelic loss both by molecular and cytogenetic approaches were transferred into a NPC cell line to determine their tumor-suppressive effects and to aid in identifying the CRs harboring potential TSGs involved in NPC tumorigenesis. Monochromosome transfer of selected chromosomes into the NPC HONE1 cell line was performed to determine whether tumor-suppressing activities for NPC mapped to chromosomes 3, 9, 11, 13, 14, and 17, as described in our previous reports [5,16–19]. By using the MMCT approach, we successfully identified CRs associated with tumor suppression in NPC and candidate TSGs mapping to these regions were subsequently studied for their role in tumor suppression. These NPC TSGs identified by the functional approaches to date are summarized in Table 1. As can be seen, the functional cloning of TSGs identified by the nude mouse assay may lead to the discovery of new functional classes of genes that are not cytotoxic *in vitro*, but are involved in different biological processes that hallmark cancer development such as genome instability and mutation, sustained proliferation, angiogenesis, inflammation, and immune evasion [20] (Fig. 2). Identified NPC TSGs can be categorized into five classes namely: cell growth and proliferation; adhesion, migration, invasion, epithelial-mesenchymal transition (EMT); metastasis; angiogenesis; transcription factors; and signaling molecules. Some of these genes belong to more than one class, as they possess multiple functions. We discuss a few examples of these TSG studies in this review.

3.1. Chromosome 3

Chromosome 3 alterations are commonly found in many human cancers [21–25]. The most frequent chromosome 3 abnormality observed involves 3p deletions, as observed both cytogenetically and by loss of heterozygosity (LOH) analyses. In NPC, multiple and extensively deleted regions in chromosome 3p are reported [26,27], which hinder the fine mapping of candidate TSGs. To obtain critical functional information to narrow down the regions of interest for identifying potential TSGs involved in NPC, the MMCT approach was utilized. A series of intact and deleted copies of human chromosome 3 derived from normal cells containing discrete interstitial deletions in the *p* arm were transferred into the tumorigenic HONE1 NPC cell line (Fig. 3). Comparisons of the tumorigenic potential of the MCHs containing these exogenous chromosome 3 fragments in nude mice identified chromosome 3p21.3 as a CR for tumor suppression in NPC [5]. A 630 kb homozygous deletion mapping to the 3p21.3 region was identified in lung, renal, and breast cancers [28]. A gene-dense 120 kb CR contains eight genes including *RASSF1A* and *BLU*(*ZMYND10*), which are candidate TSGs in several cancers, including NPC [5,28–30]. Fig. 3 shows the mapping locations of these NPC-associated chromosome 3 genes and representative functional assays.

3.1.1. ADAMTS9

Using this functional genomics mapping approach in esophageal squamous cell carcinoma (ESCC), a CR for tumor suppression was mapped to 3p14.2 and the important role of *A Disintegrin-like And Metalloprotease with ThromboSpondin type 1 motif 9* (*ADAMTS*9) was identified [31]. Subsequently, *ADAMTS9* was also identified as one of the differentially expressed genes in NPC non-tumorigenic MCHs and their matched TS cell lines. Promoter hypermethylation contributes to *ADAMTS9* gene silencing in both NPC and ESCC [31,32]. *ADAMTS9* encodes a member of a large family of 19 metalloproteases involved in maturation of precursor proteins, extracellular matrix (ECM) remodeling, cell migration, and inhibition of angiogenesis [33,34]. We subsequently evaluated the hypothesized anti-angiogenic and tumor-suppressive functions of *ADAMTS9* in NPC [35]. *ADAMTS9* activation suppressed *in vivo* tumor formation and knockdown of *ADAMTS9* resulted in the suppressive MCHs reverting to the tumorigenic phenotype. *In vivo* angiogenesis assays revealed a reduction in microvessel numbers in gel plugs injected with tumor-suppressive cell transfectants. Conditioned media from *ADAMTS9* transfectants dramatically reduced the tube-forming capacity of Human Umbilical Vein Endothelial Cells (HUVECs). By using the angiogenesis antibody array, we found that these activities were associated with decreased expression levels of the pro-angiogenic factors *MMP9* and *VEGFA*, which were consistently reduced in *ADAMTS9* transfectants. Based on the deletion patterns of the *ADAMTS9* transcript in tumors and a TS derived from the transfectants, we speculated that the tumor-suppressive activity of *ADAMTS9* was associated with the thrombospondin (TSP)