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Identification of GPR65, a novel regulator of matrix metalloproteinases using high through-put screening

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ABSTRACT

Matrix metalloproteinases (MMPs) are over-expressed in nearly all cancers. To study novel regulatory factors of MMP expression in head and neck cancer (HNC), we screened a total of 636 candidate genes encoding putative human transmembrane proteins using MMP promoter reporter in a dual luciferase assay system. Three genes GPR65, AXL and TNFRSF10B dramatically activated the induction of MMP3 expression. The induction of MMP expression by GPR65 was further confirmed in A549 and/or FaDu cells. GPR65 mediated MMP induction under acidic conditions. The AP-1 binding site in MMP3 promoter was crucial for MMP3 induction. Moreover, the A549 cells infected by recombinant adenovirus of GPR65 showed accelerated cell invasion. In conclusion, we validate that GPR65 is vital regulatory genes upstream of MMP3, and define a novel mechanism of MMP3 regulation by proton-sensing G-protein-coupled receptors.

1. Introduction

The activation of invasion and metastasis is one hallmark of cancer [1]. Matrix metalloproteinases (MMPs) are the major contributing factors during metastasis [2,3]. To date, more than 20 MMPs have been discovered [2,4]. MMPs are a family of zinc-dependent endopeptidases that degrade structural components of the extracellular matrix (ECM) to assist tumor cell metastasis. Aberrantly high level of MMPs expression has been found in multiple human tumors [2]. In line with these findings, MMPs are considered potential targets for cancer therapy. In addition to degrading extracellular matrix to promote tumor metastasis, MMPs also play important roles in controlling cell growth, inflammation and angiogenesis [3]. These functions of MMP provide new insight for cancer treatment.

Abundant evidence supports the idea that MMP expression is mainly regulated at the transcriptional level [5]. In fact, the MMP promoters contain several cis-elements, allowing for the regulation of MMP gene expression by its binding to a diverse set of transactivators, including AP-1, PEA3, Sp-1, β-catenin/Tcf-4, and NF-κB. Up to date, many regulators involved in these pathways have been reported to modulate the expressions of MMPs and contribute to tumor development [5,6].

Head and neck cancer primarily arises from the oropharynx, oral cavity, hypopharynx and larynx [7]. It is the sixth most common cancer worldwide. Stokes et al. [8] have shown that the expressions of MMP1, MMP3, MMP10 and MMP13 elevate in HNC, Patients with regional lymph node and/or distant metastases show significantly higher levels of MMP9 expression than patients without any tumor metastases [9]. The expressions of MMP9 and MMP13 are significantly different between tonsil squamous cell carcinoma and normal control tissues. Furthermore, MMP13 expression is correlated with tumor invasion, and the expression of MMP9 is correlated with nodal metastasis [10].

In this study, we aimed to identify novel regulators of MMPs that are aberrantly expressed in HNC. Using MMP luciferase reporter gene, we screened a cDNA panels encoding putative human transmembrane proteins. GPR65 was identified to activate the transcription of MMP3. Furthermore, GPR65 and other family members were further investigated on the gene expression regulation and functional analyses.

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Table 1
PCR primers used in this study.

Gene symbol	Primer (5′–3′)	Vector	Inserted site
MMP1	pF1: ggcctcgagtatgtctggactgcagtggcacaga pR1: ggcaagctttggcctttgtcttctttctcagtgc	pGL3-Basic	XhoI/HindIII
MMP12	pF12: ggcctcgagtctccaacaacaaggtaaatatctgg pR12: ggcaagcttacggatcaattcagtttactgtgttc	pGL3-Basic	XhoI/HindIII
MMP2	pF2: cggggtacctctccaacttggctctctggctatc pR2: ccgctcgagaggtcctggcaatccctttgtatgt	pGL4.17	KpnI/XhoI
MMP3	pF3: cggggtaccctgggacttgggaaacatctttca pR3: ccgctcgaggtctctatgccttgctgtcttgcct	pGL4.17	KpnI/XhoI
MMP9	pF9: cggggtaccagtgacttgcccaaggtcacata pR9: ccgctcgagtgagggcagaggtgtctgactg	pGL4.17	KpnI/XhoI
MMP10	pF10: cggggtacctgtgctagactttcgtatggcag pR10: ccgctcgagactgcccttaccttctttgtctact	pGL4.17	KpnI/XhoI
MMP13	pF13: cggggtacctttagccttcatttcatttccatagac pR13: ccgctcgagatggtgatgcctggggactgtt	pGL4.17	KpnI/XhoI
F1	pF4: cggggtacctctatcaggctttcctctaaacttt pR4: ccgctcgaggtctctatgccttgctgtcttgcct	pGL4.17	KpnI/XhoI
F2	pF5: cggggtacctttccctgtatttcaatcaggac pR5: ccgctcgaggtctctatgccttgctgtcttgcct	pGL4.17	KpnI/XhoI
F3	pF6: cggggtaccggagaatcacttgagcccaga pR6: ccgctcgaggtctctatgccttgctgtcttgcct	pGL4.17	KpnI/XhoI
F4	pF7: cggggtaccggatggattctgttcttcaacttc pR7: ccgctcgaggtctctatgccttgctgtcttgcct	pGL4.17	KpnI/XhoI
MMP3ΔAP1	pF8: aaagcaaggaagctgcgggtgat pR8: ccgcagcttccttgctttcatcc	pGL4.17	KpnI/XhoI
MMP3ΔPEA3	pF9: ctaccaagagagattaatcactgtgttg pR9: tgattaatctctcttggtagaggagaaaac	pGL4.17	KpnI/XhoI
GPR65	pF16: gtggagttcctgatgttggt pR16: gcctggagaatgtgagtgg	–	–
Beta-actin	pF17: tgacgtggacatccgcaaag pR17: ctggaaggtggacagcgagg	–	–

2. Materials and methods

2.1. MMP reporter gene plasmid construction

The promoter regions of MMP1, MMP2, MMP3, MMP9, MMP10, MMP12 and MMP13 were PCR amplified. All PCR primers are shown in Table 1. PCR fragments were inserted into pGL3-basic or pGL4.17 (Promega) using *Xho*I/*Hind*III and *Kpn*I/*Xho*I sites, respectively. The luciferase reporter gene plasmid of MMP3 promoter is named for MMP3-Luc.

In order to find crucial regulatory elements in the MMP3 promoter, a series of 5′-deletion mutants and TRE-deletion mutant of the MMP3 promoter were constructed. MMP3-Luc plasmid was used as the template, and the primers used were listed in Table 1. The MMP3 promoter region is 2037 bp (–2002 bp to +35 bp). The mutated constructs F1, F2, F3 and F4 contained −1867 bp to +35 bp, −1644 bp to +35 bp, −1500 bp to +35 bp, and −849 bp to +35 bp of the MMP3 promoter, respectively. MMP3ΔAP1 and MMP3ΔPEA3 contained MMP3 promoter deleted of the AP1 site (−64 bp to −70 bp) and the PEA3 site (−201 bp to −216 bp), respectively [5,11]. To generate MMP3ΔAP1, primers pF3 and pR8 were used to amplify the amino terminal fragment, and primers pF8 and pR3 were used to amplify the carboxyl terminal fragment. Finally, pF3 and pR3 were used to amplify the truncated mutant alleles and the amino and carboxy terminal fragments generated in the previous PCR reactions were used as the template. The truncated and deleted mutants were subcloned into pGL4.17 using the *Kpn*I/*Xho*I sites. The procedure of MMP3ΔPEA3 construction was similar to MMP3ΔAP1, except that primers pF9 and pR9 were used.

2.2. Mammalian expression vectors of human proton-sensing GPRs

GPR65 was obtained from the transmemberane protein plasmid library (see below), and the other three known human proton-sensing GPCRs (GPR132, GPR4, GPR68), were obtained from Sino-GenoMax (China).

2.3. cDNA library screening

A human cDNA library used in this study has been described previously [12]. In this study, a cDNA sub-library comprised of 636 genes encoding transmembrane proteins was used for screening. The TMHMM servers (http://www.cbs.dtu.dk/services/TMHMM-2.0/) and GO annotation (http://www.geneontology.org) tools were used for analyzing the transmembrane proteins. SignalP (http://www.cbs.dtu.dk/services/SignalP/) was used to analyze the transmembrane helixes of the N-terminal signal peptides.

2.4. Cell culture

293T, FaDu and A549 cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Neuronbc, China) supplemented with 10% fetal bovine serum. All cells were maintained at 37 °C in a humidified atmosphere containing 5% CO_2.

2.5. MMP promoter activity assay and high throughput screening

The relative luciferase activity (RLA) of MMP promoter-Luc was measured by dual luciferase activity assay as described previously [12,13]. The most sensitive MMP-Luc was selected for further screening. For screening, the dual luciferase assay and signal analysis were performed as mentioned above [12,13].

2.6. Acid stimulation and inhibitory assay

The pH of serum-free medium DMEM/F12 was adjusted to 5.9, 6.5 or 7.4 with hydrochloric acid (4 M) or sodium hydroxide (0.4 M) [14]. 293T cells were plated and transfected with four GPRs