Selective Targeting of M₃ Muscarinic Receptors: an Opportunity for Improved Treatment of Upper Gastrointestinal Carcinomas

Abstract

Upper Gastrointestinal (GI) Carcinomas are one of the most morbid forms of cancers that are inherently resistant to chemotherapeutic regimens. The G-protein coupled receptors (GPCRs) are the largest family of cell surface receptors that have recently emerged as significant modulators of cancer cell growth, survival, and metastasis. Owing to the normal physiologic role of muscarinic receptors, a major sub-class of GPCRs, many epithelial cells undergoes proliferation when exposed to acetylcholine. Since majority of the cancers are epithelial in origin, cancer cells frequently take over the phylological machinery associated with muscarinic receptors and undergo uncontrolled proliferation, avoid cell death, and exhibit invasion and migration. The M₃ sub-type of muscarinic receptors is over expressed in tumors of different types. In this review we focus on the oncogenic signaling mechanisms activated by the M₃ receptors. The manuscript highlights the importance of M₃ receptor as a potent therapeutic target for selective treatment of Upper GI Carcinomas. We also reported, for the very first time, that M₃ receptor mRNA is significantly over expressed in Upper GI Carcinoma tissue samples. In addition, we also showed that M₃-specific anti-muscarinic agents have considerable anti-cancer activity in Upper GI Cancer cells. This review serves as foundation for future studies delineating the anti-tumor effect of M₃-specific anti-muscarinic agents as single agents or in combination in Upper GI Carcinoma.

Keywords

Upper gastrointestinal cancer; M₃ muscarinic receptor; Solifenacin; Darifenacin; Oxybutynin; Microarray

Introduction

Upper Gastrointestinal (GI) Cancers (i.e. cancers of both stomach and esophagus) are the third most frequently diagnosed form of cancer world-wide [1]. Latest GLOBOCAN 2012 global epidemiological data indicate that Upper GI Carcinoma accounts for approximately 1.12 million deaths annually making it the second most morbid form of cancer [1]. In addition, as per the Surveillance, Epidemiology, and End Results (SEER) data published by National Cancer Institute the incidence and mortality rates for gastric cancers in US have reduced marginally, however, there has been no change in these parameters for esophageal cancers for the last three decades.

Upper GI Carcinoma as an aggressive malignancy characterized by inherent resistance to current chemotherapeutic regimens, high rates of disease recurrence, tumor metastasis and poor patient survival [2-6]. Multiple potent oncogenic molecular pathways driven by the epidermal growth factor receptor (EGFR), human epidermal growth factor receptor 2 (HER-2), v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog (K-RAS), and Aurora kinase A (AURKA) pathways are constitutively overactive in Upper GI Carcinomas [7-11]. Therefore, development and characterization of novel chemotherapeutic regimens based on the molecular makeup of Upper GI Carcinomas are urgently needed to reduce patient mortality and morbidity.

The G-protein coupled receptors (GPCRs) are a super-family of receptor proteins that account for the largest number of cell surface receptors expressed by any human cell [12]. GPCRs modulate multiple signaling pathways that play a vital role in maintaining normal cellular physiology and homeostasis. GPCRs mediatepotentialsensing mechanisms that govern normal physiological responses associated with cell proliferation, survival and motility [13]. Unfortunately, aberrant and deregulated activity of GPCRs has been observed in various types of cancers and is associated with malignant transformation due
to overactive oncogenic signaling mechanisms resulting in tumor growth, drug resistance, angiogenesis and metastasis [13]. The Muscarinic Cholinergic Receptors (mAChRs) are one of the five major families of GPCRs and consist of five distinct subtypes (M₁, M₂, M₃, M₄, and M₅) [12]. The M₂, M₃, and M₅ mAChRs are inhibitory in function and interact with Gαi/Go subunit of G-proteins. Their activation results in reduced synthesis of cAMP from cellular ATP within the cells [15]. Studies conducted in various animal models have shown that M₃ and M₅ mAChRs are expressed in normal epithelial cells of the Upper GI tract [17-19]. Majority of the cancers are derived from epithelial cells that have undergone malignant transformation. Therefore, most of them exhibit aberrant muscarinic signaling in the form of over expressed mAChR sand/or enhanced activation by acetylcholine (ACh) secreted from autocrine and paracrine sources.

The M₃ mAChR is the subtype majorly over expressed in lung, skin, colon, gastric, pancreatic (subtype not identified), breast, ovarian, prostate and brain cancers [20-30]. The M₃ mAChR has been induced to produce multiple signaling pathways associated with cellular growth, survival, inflammation, angiogenesis, invasion and migration in different types of cancer cells. Some of the key oncogenic cellular mechanisms activated by M₃ receptors in cancer cells include the EGFR/MAPK pathway (growth and proliferation), PI3K/Akt pathway (pro-survival and anti-apoptosis), β-catenin/Wnt pathway (invasion and migration) and NFkB pathway (pro-inflammatory and chemotactic).

The proliferative MAPK pathway and the pro-survival PI3K/ Akt pathway are frequently activated by cell surface localized RTKs. EGFR is one such RTK that is transactivated by M₃ receptors through paracrine acetylcholine signaling mechanisms in colon cancer cells [31]. Transactivation of EGFR by M₃ receptor stimulates the MAPK pathway resulting in colon cancer cell proliferation [32]. In small-cell lung cancer (SCLC) inhibition of M₃ receptor with Darifenacin (an M₃-specific antagonist) reduced the activity of MAPK pathway and suppressed overall tumor growth in vitro and in vivo [33]. Specific inhibition of M₃ receptor with 4-DAMP (an M₃-specific antagonist) and M₃ receptor knock down suppressed phosphorylation of MAPK in MCF-7 breast cancer cells [27]. In addition, M₃ receptor mediated activation of PI3K/Akt pathway has also been indicated to play a vital role in inducing growth and proliferation of astrocytoma cells [34].

The M₃ receptor signaling is particularly pronounced in the lungs where M₃ receptor expression has been correlated with tumor metastasis and poor survival rates in patients with non-small-cell lung cancer (NSCLC) [20]. The M₃ receptors are indicated to induce invasion and migration of NSCLC cancer cell lines by enhancing the expression and activity of matrix metalloproteinase 9 (MMP9) through the PI3K/Akt pathway [20]. In melanomas, M₃ receptor expression has also been correlated with invasion and migration [22]. The Wnt/β-catenin signaling pathway plays an important role in inducing invasion and metastasis of cancers. The regulatory effect of M₃ receptor on Wnt/β-catenin signaling pathway was determined by Raufmann et al. in Apc⁺/− mice that were knocked out for M₃ receptor gene [35]. Their data indicated that M₃ receptor knockout Apc⁻/⁻ mice exhibited reduced activity of β-catenin as evidenced by decreased nuclear localization. Furthermore, M₃ receptor can mediate drug resistance since it has been shown to prevent induction of P53 pro-apoptotic protein in Chinese hamster ovary (CHO) cells following treatment with a DNA-damaging agent [36]. The M₃ receptors have also been implicated in promoting angiogenesis by inducing nitric oxide synthesis, prostaglandin E₂ (PGE2) production and VEGF expression in breast cancer cells [37-39]. Chronic Inflammation is an important precursor for carcinogenesis. The nuclear factor kappa B (NFκB) pathway plays a critical role in regulating expression of genes mediating inflammation. Carbanchol induced M₃ receptor activation has been associated with increased activation of NFkB pathway in human astrocytoma cells and up-regulation of cyclooxygenase-2 (COX-2) enzyme mediated PGE2 synthesis in colon cancer cells [40,41]. Together, NFkB and PGE2 can mediate chronic inflammatory response resulting in sustained cellular damage that can ultimately induce malignant transformation. These observations suggest that M₃ receptor function is frequently up-regulated in cancer cells and it can play an important role in the overall progression of various types of cancers. However, there is paucity in scientific literature with respect to the status of M₃ receptor expression and function in human Upper GI Carcinomas. At present the status of muscarinic receptor expression in normal human esophageal epithelial cells and cancer cell lines is unknown. In addition, there is only one study published by Kodaira et al. [25] that demonstrated the presence of M₃ receptors in various human gastric cancer cell lines [25]. Therefore, to further establish the clinical relevance of M₃ receptor expression for the treatment of Upper GI Carcinomas we first investigated the presence of mRNA for various muscarinic receptors (M₁-M₅) in tumor tissue samples from patients. We mined the publically available NCBI GEO database for microarray data associated with normal and cancerous Upper GI tissue samples. The microarray data (Figure 1A & 1B) indicate that mRNA expression of M₃ receptor is significantly high in both gastric (normal gastric tissue: 3.429 ± 0.3068, N=7; gastric cancer: 11.17 ± 2.689, N=22; p < 0.01) [42] and esophageal (normal esophageal tissue: 5.510 ± 0.1795, N=17; esophageal cancer: 6.953 ± 0.1373, N=17; p < 0.01) [43] cancers. Our data analysis did not show any significant increase in the mRNA expression for M₁, M₂, M₄ and M₅ receptors in the aforementioned microarray data sets. We also used various M₃ receptor specific anti-muscarinic agents (solifenacin,
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Discussion and Conclusion

The aberrant GPCR signaling network plays a pivotal role in activating multiple oncogenic mechanisms involved in cancer development and progression. The M₃ mAChR is one of the major GPCRs that are up-regulated in different types of cancers and it induces growth, survival, drug resistance, angiogenesis, invasion, migration and inflammation associated signaling mechanisms. The microarray data indicate that M₃ receptors are up-regulated in Upper GI Carcinomas and the cell viability data shows that M₃-specific anti-muscarinic agents inhibit Upper GI Cancer cell viability. At present, promising clinically approved M₃-specific anti-muscarinic drugs are available that are well tolerated and exhibit minimal side effects. Since Upper GI Carcinomas exhibit resistance to multiple chemotherapeutic agents it is possible that a combination regimen consisting of a first line chemotherapeutic agent like Cisplatin, Docetaxel, or 5-Fluorouracil (5-FU) with M₃-specific anti-muscarinic agents will show enhanced antitumor activity. Overall, based on M₃ receptor function, expression and availability of M₃-specific anti-muscarinic agents we suggest that future studies should be conducted to determine the therapeutic value of selective targeting of M₃ receptors for the treatment of Upper GI Carcinomas.

Methods

Cell Culture and Pharmacologic reagents: AGS Upper GI Cancer cell line was maintained as a monolayer culture in F12 Media (Gibco) cell culture medium supplemented with 10% (v/v) FBS (Gibco). Solifenacin and Oxybutynin stock solutions were prepared in water and further diluted in cell culture media. Darifenacin stock solution was prepared in dimethyl sulfoxide (DMSO).

MTT Cell Viability Assay: AGS cells were seeded at 2000 cells/well into 96-well plates. The plated cells were treated with Solifenacin (0.5–30 μM), Darifenacin (50–400 nM), or Oxybutynin for cell viability assay. Darifenacin stock solution was prepared in dimethyl sulfoxide (DMSO).

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Oxybutynin (0.5–100 μM) and incubated for 72 hours at 37°C and 5% CO₂ level in an incubator (Panasonic Healthcare Co.). Subsequently, 25 μL of MTT reagent (5mg/mL in phosphate buffered saline pH 7.2 or PBS) was added into each well and the plates were incubated for 4 hours. The violet colored formazan crystals formed in each well were dissolved in 100 μL of DMSO. The absorbance of dissolved formazan crystals was measured at 540nm in a plate-reader (BioTek Instruments, Inc.). The intensity of the signal is a measure of overall cellular metabolic activity and an indicator of viable cells present at the end of the treatment.

**Statistical Analysis:** Data are presented as means ±SEM. All cell viability experiments were done in triplicates. One-way ANOVA with Tukey post hoc analysis was used to show statistical difference between control and treatment groups. Statistical analyses were done with Graph Pad Prism 5 software (Graph Pad Software, Inc.). For microarray RNA expression analysis T-test was done to determine statistical difference between normal tissue and tumor tissue samples. The p value of ≤0.05 was considered statistically significant. Statistically significant differences are marked in the Figures; *p<0.05, **p<0.01.

**References**


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