Published in final edited form as:

Ann Surg Oncol. 2011 May ; 18(5): 1506–1511. doi:10.1245/s10434-010-1488-z.

Resveratrol Induces Notch2-mediated Apoptosis and Suppression of Neuroendocrine Markers in Medullary Thyroid Cancer

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Abstract

Background—Currently, complete surgical resection is the only curative option for MTC. Previous work has shown the Notch pathway is a potent tumor suppressor in MTC and that Resveratrol activates the Notch pathway in carcinoid cancer, a related NE malignancy. In this study, we hypothesized that the effects observed on carcinoid cells could be extended to MTC.

Methods—MTC cells treated with varying doses of Resveratrol were assayed for viability using the MTT assay. Western blot analysis for Achaete-Scute Complex-Like 1 (ASCL1), chromogranin A (CgA), full-length and cleaved caspase 3, and poly-ADP ribose polymerase (PARP) was performed. Quantitative real-time PCR (qPCR) was used to measure relative mRNA expression.

Results—Treatment with Resveratrol resulted in growth suppression and an increase in the cleavage of caspase-3 and PARP. A dose-dependent inhibition of ASCL1, a NE transcription factor, was observed at the protein and mRNA levels. Protein levels of CgA, a marker of hormone secretion, were also reduced after treatment with Resveratrol. A dose-dependent induction of Notch2 mRNA was observed using qPCR.

Conclusions—Resveratrol suppresses in vitro growth, likely through apoptosis, as demonstrated by cleavage of caspase-3 and PARP. Furthermore, Resveratrol decreased NE markers ASCL1 and chromogranin A. Induction of Notch2 mRNA suggests that this pathway may be central in the anti-MTC effects observed.

Medullary thyroid cancer (MTC) is derived from the calcitonin-producing parafollicular cells. This cancer accounts for 3–5% of thyroid cancer cases, though up to 14% of thyroid cancer deaths [1]. While early total thyroidectomy may be potentially curative for familial cases of MTC, sporadic MTC accounts for approximately 75% of cases. Surgical resection in the patient population, however, results in a recurrence rate of almost 50%. Additionally, patients with MTC suffer from debilitating endocrinopathies, related to excessive hormone secretion. The acidic glycopeptide chromogranin A (CgA) is typically co-secreted with these hormones and is used as a clinical marker of the disease [2–4]. Traditional adjunctive therapies, including chemotherapy and radiation, have shown limited efficacy, highlighting the need for novel therapeutic options [5–9].

While the role of Notch as an oncogene is well defined in breast, colorectal, prostate, and pancreatic adenocarcinoma, the Notch signaling pathway has been shown to have a

paradoxical tumor suppressive role in neuroendocrine (NE) cancer types [10]. The Notch genes encode transmembrane receptors that regulate cellular differentiation, development, proliferation, and survival in many contexts. Upon binding of a ligand, a series of proteolytic cleavage steps occurs and the Notch Intracellular Domain (NICD) is released. This fragment translocates to the nucleus to activate a variety of target genes. In MTC cells, overexpression of Notch1 has been shown to inhibit growth and suppress Achaete-Scute Complex-Like 1 (ASCL1), an important helix-loop-helix transcription factor that regulates the NE cancer phenotype and has been correlated with poor prognosis in NE tumors [11]. ASCL1 has been shown to play a particularly important role in the thyroid as ASCL1 null mice fail to develop parafollicular cells [12,13]. Additionally, through ASCL1, Notch activation has been shown to suppress the levels of CgA [3,4].

Work by our group and others has validated the Notch pathway as a tumor suppressing pathway in MTC. Overexpression studies showed that Notch can suppress growth as well as ASCL1 expression. Pharmacologic induction of the Notch pathway was additionally shown to be a potent anti-MTC strategy *in vitro* [14–16]. This finding was subsequently confirmed *in vivo* [17]. Thus, drugs that target the Notch pathway are candidates for treatment of MTC.

Recently, our group developed a quantitative high throughput screen to identify Notch activating compounds. Pinchot et al. identified and validated Resveratrol as a potential Notch activator in gastrointestinal (GI) and pulmonary carcinoid cells. Resveratrol treatment was shown to inhibit the growth of carcinoid cells both *in vitro* and *in vivo* while suppressing the expression of ASCL1, CgA, and serotonin [18].

Resveratrol is a dietary polyphenol found in the skins of grapes and peanuts. A growing body of evidence suggests that Resveratrol may delay the onset of a variety of illnesses, including cancer, cardiovascular disease, and ischemic injuries [19]. High doses of Resveratrol *in vivo* did not reveal any harmful alterations in hematology, clinical chemistry, and organ histopathology [20]. Currently, Phase I/II clinical trials of Resveratrol are underway for the treatment of colorectal cancer and lymphoma [21].

In this study, we extended our analysis of Resveratrol from carcinoid to MTC. We show that Resveratrol suppresses growth, induces apoptosis, reduces ASCL1 and CgA expression, and increases Notch2 mRNA in MTC cells. These results are the first description of a Notch2 activator in MTC and suggest that Resveratrol may be a potential therapeutic option for MTC.

METHODS

Cell culture

Human MTC (TT) cells were obtained from American Type Culture Collection (Manassas, VA) and maintained in RPMI 1640 (Invitrogen Life Technologies, Carlsbad, CA) supplemented with 16% FBS (Sigma), 100 IU/ml penicillin, and 100 µg/ml streptomycin in a humidified atmosphere of 5% CO₂ in air at 37°C.

Cell Proliferation Assay

TT cell proliferation was measured using the 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide (MTT) assay as previously described [13]. Cells were seeded in quadruplicate into 24-well plates and were incubated overnight to allow cell adhesion. After incubation, cells were treated 0–25 µM Resveratrol (Biomol International, Plymouth Meeting, PA). Treatment medium was changed every two days with new dilutions of drug. To perform the MTT assay, cells were washed with phosphate buffered saline (PBS) and incubated in 250 µl of serum-free RPMI 1640 containing 0.5 mg/ml MTT for 4 hours. After

incubation, 750 µl of dimethyl sulfoxide (Fischer Scientific, Pittsburg, PA) was added to each well and mixed thoroughly. Absorbance at 540 nm was measured using a spectrophotometer (µQuant; Bio-Tek Instruments, Winooski, VT) and plotted as an average $\pm$ standard error of the mean (SEM).

Immunoblotting

TT cells were incubated overnight to allow cell adhesion. After incubation, cells were treated for 2 days with varying doses of Resveratrol. Total protein was harvested as previously described and quantified using the BCA Protein Assay Kit (Thermo Scientific, Waltham, MA) according to the manufacturer's instructions [13]. Denatured cellular extracts were resolved on a 10% SDS-PAGE gel, transferred onto nitrocellulose membranes (Bio-Rad Laboratories, Hercules, CA), and blocked in milk. Membranes were incubated overnight in primary antibodies with the following dilutions: 1:2000 for mammalian achaete scute homologue-1 (MASH1) for ASCL1 (BD PharMingen, San Diego, CA); 1:500 for chromogranin A (CgA) (Zymed Laboratories, San Francisco, CA); 1:1000 for poly-ADP ribose polymerase (PARP), caspase 3, and cleaved caspase 3 (cl-caspase 3) (Cell Signaling Technology, Beverly, Mass); and 1:10,000 for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Trevigen, Gaithersburg, MD). Membranes were then incubated in appropriate amounts of horseradish peroxidase conjugated goat anti-mouse or anti-rabbit antibody (Cell Signaling Technology), which were detected using Immun-star (Biorad) or Supersignal West Femto (Pierce Protein Research Products, Rockford, IL) kits, according to the manufacturer's instructions.

Quantitative Real-time PCR

To quantify mRNA expression, we used quantitative real-time PCR (qPCR). Following treatment as above, total RNA was isolated using RNeasy Mini-kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. The iScript cDNA Synthesis Kit (Bio-Rad) was used to synthesize cDNA using 2 µg of RNA per sample. Amplification and detection of PCR products were performed using iQ SYBR Green (Bio-Rad). The following PCR primer pairs were used: Notch2 (5'-TGTGACATAGCAGCCTCCAG-3' and 5'-CAGGGGGCACTGACAGTAAT-3'), ASCL1 (5' TCC CCC AAC TAC TCC AAC GAC 3' and 5' CCC TCC CAA CGC CAC TG 3'), and GAPDH (5'-ACCTGCCAAATATGATGAC-3' and 5'-ACCTGGTGCTCAGTGTAG-3'). The following conditions were used for PCR amplification: 95°C for 3 min, 35 cycles of: 95° for 30 sec, 60° for 25 sec, 72° for 30 sec, followed by 95° for 1 min and 55° for 1 min. All PCR reactions were done in triplicate. Threshold cycles, Ct, were measured with iCycler Real-Time PCR Instrument and iCycler Software (Bio-Rad). Target gene expression levels were calculated using the ΔC_t method with the formula $2^{(C_t(GAPDH) - C_t(target))}$, as described in Real-Time PCR Applications Guide (Bio-Rad). Expression in treatment groups was compared to control expression and plotted as an average $\pm$ SEM.

Statistical Analysis

Statistical analysis was conducted using one-way analysis of variance (ANOVA) (SPSS software, version 10.0; SPSS Inc., Chicago, IL). A p value of less than 0.05 was considered significant.

RESULTS

Resveratrol Inhibits MTC proliferation

Human MTC cells were treated for up to 6 days with increasing doses of Resveratrol and the MTT assay performed after 4 and 6 days of treatment. Compared to control, 25 µM, 50 µM,

and 100 μ M Resveratrol treatments for 4 days reduced growth by 5%, 8.9%, and 16.4%, respectively ($P < 0.05$). Treatment for 6 days resulted in a 19%, 38%, and 38% suppression of growth, respectively ($P < 0.002$) (Fig. 1).

Resveratrol Induces Cleavage of Apoptotic Markers

To explore the mechanism of Resveratrol-induced growth suppression, we examined cleaved caspase 3 (cl-caspase 3) and cleaved poly-ADP ribose polymerase (cl-PARP) using Western blotting. The intrinsic and extrinsic apoptotic pathways both result in the cleavage of caspase 3, a critical protease that cleaves other apoptotic proteins such as poly-ADP ribose polymerase (PARP) to promote cell death. Thus, increased cleavage of caspase 3 and PARP suggest activation of apoptotic pathways [22–24]. We found that treatment with Resveratrol resulted in increased levels of both cl-caspase and cl-PARP. We also probed for full-length caspase 3 and PARP to verify that increased cl-caspase 3 and cl-PARP are attributable to proteolytic cleavage as opposed to an increase in total protein expression (Fig. 2).

Resveratrol Suppresses Markers of MTC

We hypothesized that MTC growth inhibition would be associated with a change in NE markers. Thus, we examined levels of ASCL1 and CgA, both important markers of MTC [2–4,11]. Using Western blotting, we found that treatment of Resveratrol resulted in a dose-dependent reduction in CgA after 2 days of treatment (Fig. 3a). This finding is notable as CgA is co-secreted with bioactive hormones and used as a clinical marker of disease [2,4].

ASCL1 is transcription factor that is highly expressed in MTC and is strongly correlated with levels of CgA [13]. Furthermore, elevated ASCL1 protein is associated with poor prognosis in related neuroendocrine tumors (NETs) [24,25]. Thus, we performed western blot analysis for ASCL1 and found that Resveratrol suppressed ASCL1 protein levels after 2 days of treatment (Fig. 3a).

As ASCL1 suppression could occur at a number of levels, we performed qPCR for ASCL1 mRNA. Treatment of Resveratrol resulted in a dose-dependent inhibition of ASCL1 mRNA levels. Notably, a 70% reduction in ASCL1 mRNA levels was achieved with 100 μ M treatment of Resveratrol ($P < 0.001$) (Fig. 3b).

Resveratrol Induces Notch 2 mRNA

Previously studies have attributed growth inhibition, induction of apoptosis, and suppression of MTC markers to Notch pathway activation [27]. Thus, we investigated the effects of Resveratrol on Notch2 mRNA expression, using qPCR. Treatment of Resveratrol resulted in a dose-dependent induction of Notch2 mRNA expression, with a significant 15-fold induction with the 100 μ M dose ($P < 0.001$) (Fig. 4). We also examined mRNA expression of other Notch isoforms and did not observe an induction (data not shown).

DISCUSSION

MTC is more difficult to treat and results in an increased mortality, compared to other forms of thyroid cancers [28]. In addition to a significant mortality rate, MTC is associated with debilitating symptoms such as dyspnea, dysphagia, diarrhea and flushing, associated with increased levels of hormone secretion and CgA [2,10]. Since surgery is the only curative option and traditional chemotherapy and radiation have limited efficacy, novel therapeutic approaches are necessary.

The Notch1 pathway has been validated as a tumor suppressor in MTC and has been shown to suppress ASCL1 and CgA expression [13]. Previous studies by our group and others have shown that targeting the Notch1 pathway *in vitro* and *in vivo* is a potential anti-MTC strategy [16,17]. Importantly, there appears to be significant overlap in the molecular targets of Notch1 and Notch2 [29]. Recently, Notch2 has been shown to induce many of the same biological effects as Notch1 in tumors of NE origin. In GI and pulmonary carcinoid cells, blocking Notch2 with small interfering RNA has been shown to rescue the Resveratrol-induced ASCL1 suppression [18]. We extend our group's previous work with Resveratrol in this paper by examining the effects of Resveratrol in MTC.

We show here that Resveratrol is able to significantly suppress the *in vitro* proliferation of MTC cells after just 4 days of treatment. Although the degree of growth suppression was modest, the effect on neuroendocrine markers was substantial. Additionally, we demonstrate an increased cleavage in apoptotic markers, suggesting that induction of apoptosis is the mechanism of growth inhibition. Western blot analysis revealed suppression of CgA, a marker of hormone secretion. These data suggest the Resveratrol is capable of suppressing bioactive hormones implicated in the debilitating edocrinopathies associated with MTC. We show that Resveratrol suppresses the protein and mRNA levels of ASCL1, a transcription factor that is critical in normal parafollicular cell development. ASCL1 is also correlated with poor prognosis in NETs and is a known mediator of CgA expression in MTC [1,4,10,12,13,30]. We additionally show that these changes are associated with an induction of Notch2, a tumor suppressing pathway in MTC.

In this study, we demonstrate the anti-MTC potential of Resveratrol and provide the first description of a pharmacologic Notch2-inducing compound in MTC. Resveratrol is a naturally occurring compound with an established safety profile [19–21]. Given the demonstrated efficacy *in vitro* and known *in vivo* safety profile, Resveratrol is worthy of additional pre-clinical investigation.

Acknowledgments

Grants: Department of Surgery T35 Short Term Training Grant DK 062709-0401 (MT), Howard Hughes Medical Institute (MRC), NIH – RO1 CA121115 (HC), NIH – RO1 CA109053 (HC), American College of Surgeons: George H. A. Clowes Jr. Memorial Research Career Development Award (HC), Carcinoid Cancer Foundation Research Award (HC)

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FIG. 1. Resveratrol inhibits medullary thyroid cancer (MTC) cell proliferation. TT cells were treated with increasing doses of Resveratrol for up to 6 days and cell viability was measured using the 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide (MTT) assay. A dose-dependent, statistically significant reduction in MTC viability was observed ($P < 0.05$).

FIG. 2.

Resveratrol treatment induces markers of apoptosis in MTC cells. TT cells were treated with Resveratrol for 2 days. Cell lysates were prepared and probed for apoptotic markers using Western blotting. Cleavage of poly-ADP ribose polymerase (PARP) was observed with Resveratrol treatment. Additionally, Resveratrol caused a dose-dependent increase in the cleavage of caspase 3. Glyceraldehyde 3-Phosphate – Dehydrogenase (GAPDH) is presented as a loading control.

FIG. 3. Resveratrol suppresses markers of MTC. **a** Resveratrol inhibits Achaete-Scute Complex-Like 1 (ASCL1) and chromogranin A (CgA) protein expression. Treatment for 2 days with increasing doses of Resveratrol suppressed the protein expression of ASCL1 and CgA, two well known markers of MTC. GAPDH is presented as a loading control. **b** Resveratrol inhibits ASCL1 mRNA expression. Treatment for 2 days with Resveratrol resulted in the dose-dependent suppression of ASCL1 mRNA expression. Notably a 70% reduction in mRNA expression was observed with 100 μM treatment of Resveratrol ($P < 0.001$). Expression levels were normalized to GAPDH from the respective samples and plotted vs. control expression.

FIG. 4.

Resveratrol induces Notch2 mRNA expression. MTC cells were treated for 2 days with increasing doses of Resveratrol and Notch2 mRNA expression analyzed by qPCR. A dose-dependent induction in the mRNA level of this important tumor suppressor was observed ($P < 0.001$). Expression levels were normalized to GAPDH from the respective samples and plotted vs. control expression.