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TRAIL DECOY RECEPTOR INACTIVATION AS A BIOMARKER OF RESPONSE IN CERVICAL CANCER

Dr. Sandeep Kumar Mittan
Research officer RML Hospital, New Delhi.110001

ABSTRACT

Cervical Cancer (CC) is the third most common cancer in women worldwide. When invasive cancer is diagnosed thecure rate is low resulting in high mortality. The treatment response of invasive and metastatic CC remains unpredictable. There is an unmet need for targeted therapies in CC based on genetic/epigenetic pathways. Towards this goal, utilizing the published evidence of inactivation of tumor necrosis factor receptor superfamily genes (TNFRSF10C/DcR1, and TNFRSF10D/DcR2) play a role in apoptosis in CC, the present proposal seeks to further investigate this pathway to develop clinically significant therapeutic approach to stratify patients for TRAIL-combination therapy. We will investigate the molecular mechanisms regulated by TNFRSF10C and TNFRSF10Dinactivation in TRAIL-mediated apoptosis in CC.Using a panel of well characterized CC cell lines, we will address (i) the contribution of relative roles of TNFRSF10Cand TNFRSF10D gene inactivation in vitro in TRAIL-combination therapy in inducing apoptosis by depletionand over-expression approaches, and (ii) determine the functional role of inactivation of these genes intumoricidal activity of TRAIL-combination therapy in CC tumor xenograft model. Success of these research studies will provide an essential platform for the development of a new molecular based test for stratification of CC for TRAIL targeted therapy.

RESEARCH PROPOSAL

Objective of the Study: 1) To provide an essential platform for the development of a new molecular based test for stratification of Cervical cancer (CC) for TRAIL targeted therapy.
2). To Developed an opportunity to explore strategies to test decoy receptor gene inactivation as a biomarker of response to Apo2L/TRAIL-combination therapy.

RESEARCH PLAN

***Significance*:** Cervical Cancer (CC) is the third most common cause of cancer-related mortality in women world-wide, with >300,000 deaths per annum(Waggoner, 2003). Over 90% of CCs carry high-risk human papilloma virus (HRHPV) DNA sequences. Deregulated expression of viralE6 and E7 oncogenes results in clonal expansion and accelerated accumulation of genetic alterations(Munger and Howley, 2002). Treatment of CC using platinum based derivatives followed by radiation exhibit considerable differences in response, where a sizable proportion of advanced tumors acquire resistance (Nezhat et al., 2004). Vaccination against HRHPV will not

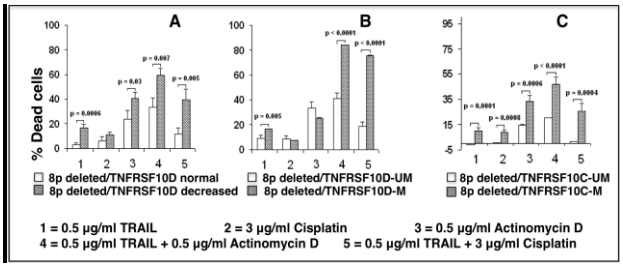
prevent CC in women who are already infected.Thus, there is an important need for more effective clinical interventions in CC. Recently, biologically targeted agents have been shown to significantly improve an overall survival in combination therapies (Tewari and Monk, 2014). The roles of genetic and epigenetic alterations in treatment response of CC are largely unexplored(Mitra et al., 1994; Narayan et al., 2007). Despite the availability of large genomic data, the stratification of CC into subclasses for response to treatment remains elusive(Ojesina et al., 2014). Thus, the major issue in CC is to uncover genetic pathways for developing specific targeted therapies. Using SNP array Dr. Murty's laboratory identified a minimal region of deletion (MRD) at 8p12-21.3 containing four tumor necrosis factor related super family (TNFRSF) receptor genes: two death receptors (DRs)(*DR4*/*TNFRSF10A* and *DR5*/*TNFRSF10B*) and two decoy receptors (DcRs)(*DcR1*/*TNFRSF10C* and *DcR2*/*TNFRSF10D*)(Narayan et al., 2016). The DRs expressing on cell surface are critical regulators of extrinsic apoptotic pathway in TNF-related apoptosis-inducing ligand (TRAIL)/APO2L-mediated cell death. Binding TRAIL to their cognate DRs result in death inducing signaling complex (DISC) formation and initiates activation of proteases caspase 8 and 10, thereby driving downstream effector caspase activation and apoptosis. TRAIL triggers apoptosis in cancer cells while sparing normal cells. Despite this specificity, many cancers exhibit resistance to TRAIL. Non-functional DcRs compete with DRs in binding to TRAIL. Therefore, over expressing antagonistic DcRs inhibit TRAIL-induced apoptosis (Morizot A et al., 2011). But TRAIL-resistance in tumor cells could not be predicted just by over expressing DcRs alone and the molecular mechanisms of sensitivity remains unclear (LeBlanc and Ashkenazi, 2003). Cellular resistance to TRAIL also result from large variety of events such as defects in DISC formation and other distal signaling molecules such as c-FLIP, IAP, and XIAP. Dr. Murty's laboratory has demonstrated that promoter hypermethylation-mediated down-regulated transcription of *TNFRSF10C* and *TNFRSF10D* genes in CC cell lines result in a high synergisticapoptotic response to TRAIL-combination drug therapies (Fig. 1). It has been shown that the cervical cancer cell lines harboring epigenetic inactivation of TRAIL decoy receptors effectively activate downstream caspases suggesting a critical role of inactivation of these genes in efficient execution of extrinsic apoptotic pathway and therapy response. Therefore, these findings shed new light on the role of genetic/epigenetic defects in TRAIL decoy receptor genes in the pathogenesis of CC and provide an opportunity to explore strategies to test decoy receptor gene inactivation as a biomarker of response to Apo2L/TRAIL-combination therapy. Based on these findings, we propose to investigate the precise role of DcR inactivation in TRAIL apoptotic response*in vitro* and *in vivo*. Such an understanding will allow stratification of patients for targeted TRAIL therapy that may transform the current modalities of intervention in CC.

Innovation: Activating tumor specific apoptotic pathways is a major goal in cancer therapies. Use of TRAIL and TRAIL receptor agonists singly or with drug combinations is currently emerging as a promising cancer apoptosis-targeted therapy

(Tolcher et al., 2007). The role of genetic/epigenetic alterations in death and decoy receptors to TRAIL response has not been fully understood. The outcome of the proposed studies will provide a scientific rationale for selective use of combination agents that activate TRAIL pathway and a potential

prognostic value in the prediction of TRAIL response, which will be a complete paradigm shift in CC treatment.

Figure 1*. Apoptosis analysis by flow cytometry in cervical cancer cell lines after exposure to TRAIL-combination drugs in relation to decoy receptor status and 8p deletion.*



Approach

i) *Investigating relative roles of TNFRSF10D and TNFRSF10C in inducing TRAIL-mediated apoptosis in vitro in CC cell lines.*

The mechanisms of differential TRAIL response in tumors are not clear (Johnstone et al., 2008; Spencer et al., 2009). Based on the reported findings, we propose to study DcR inactivation as a potential therapeutic target for TRAIL response examining the relative roles of *TNFRSF10D* and *TNFRSF10C* by depleting or restoring these genes.Since *TNFRSF10C* and *TNFRSF10D* inactivation predicts TRAIL response, we hypothesize thattheir suppression in resistant cell lines render sensitivity and restoration in sensitive cell lines will acquire resistance.To test this, we will employ gene depletion and over-expression approaches to determine the contribution of DcR genes and their synergistic affect on apoptotic response.

In knockdown experiments, we will employ siRNA and CRISPR/Cas9 approaches to generate transient and stable transfectants, utilizing cervical cancer cell lines MS751 and SW756. Both exhibit 8p deletion and are resistant to TRAIL-cisplatin induced apoptosis(Narayan et al., 2016). MS751 showed no evidence of methylation or down-regulated expression of both DcRs, while SW756 exhibit only*TNFRSF10C* methylation and down-regulated expression. We will transfect either *TNFRSF10D* or *TNFRSF10C* alone or together, initially using gene specific siRNA along with scramble control. To generate stable transfections we will utilize CRISPR/Cas9 with or without luciferase labeling. Endogenous and the induced down-regulated expression will be monitored by western blot and flow cytometry. Cells sensitivity to recombinant human TRAIL (rhTRAIL) in combination with cisplatin, actinomycin D or radiation for cell death will be

assessed. siRNA/CRISPR-Cas9-mediated down-regulation of *TNFRSF10D* and/or *TNFRSF10C* should restore sensitivity to TRAIL if they are functional. To examine the inactivation issue further, we will employ forced expression of DcR genes to test the hypothesis that over expression results in resistance to TRAIL. We will transfect pcDNA3.1/Zeo expression vectors with the aim to obtain high expression of *TNFRSF10D* and/or *TNFRSF10C*. We will utilize cell lines ME-180 and C-33A that exhibit promoter methylation-mediated inactivation of *TNFRSF10C* and *TNFRSF10D*. ME-180 showed highest apoptosis to TRAIL-combination therapy while C-33A cell line was relatively less sensitive(Narayan et al., 2016). The cell lines with ectopic expression of *TNFRSF10D* and *TNFRSF10C*, separately or in combination, should become resistant if their inactivation is critical for TRAIL-combination treatment compared to parental cell lines. These functional assays will confirm that inactivation of one or both DcRs determine the response to TRAIL, and will serve as indicators for stratifying CC patients for effective therapy. Since selective induction of apoptosis via TRAIL-R1 pathway has been reported using an agonistic humanized MAb mapatumumab, we will simultaneously test DR4/DR5- specific monoclonal antibodies if a better response can be achieved compared to rhTRAIL.

Since DRs and DcRs dynamically alter their localization in tumor cells upon exposure to TRAIL (Zhang et al., 2000), and $cFLIP_L$ (CFLAR) regulates apoptosis in primary keratinocytes (Leverkus et al., 2000; Wachter et al., 2004), we will also examine the affect of knockdown/restoration of *TNFRSF10D* and *TNFRSF10C* on the levels and surface localization of DRs and activation of caspases 8, 9, 3 and c-$FLIP_L$ by RT-PCR and western blot analysis. The readout of these experiments will be whether (i) inactivation of *TNFRSF10D* or *TNFRSF10C* alone is sufficient to sensitize the cells to TRAIL therapy, and (ii) inactivation of both genes will have synergistic affect.

ii). Effects of tumoricidal activity of TRAIL combination therapy in relation to TNFRSF10D and TNFRSF10C suppression in cervical cancer tumor xenograft model.

We will ask the potentiation of drugs to tumor cells *in vitro*can be replicated *in vivo* due to enhanced toxicity and modified pharmacokinetics, using a xenograft model. Towards this, we will examine the tumoricidal effects of TRAIL, cisplatin, TRAIL+cisplatin on the same parental and modified cell lines outlined in *in vitro* studies to test the ability of *TNFRSF10D* and *TNFRSF10C* inactivation in TRAIL-induced tumor regression. Using NOD-SCID mice, we will measure growth and metastasis in subcutaneous xenografts and quantify lung colonization following vein injection. Questions we ask are: a). Will mice administered TRAIL alone, cisplatin alone, or combination of both, reduce tumor size in cell lines depleted *TNFRSF10D* and *TNFRSF10C* genes alone or together. b). Will mice administered TRAIL alone, cisplatin alone, or combination of both, with reconstituted *TNFRSF10D* and *TNFRSF10C* genes alone or together will increase the tumor growth. Primary questions that will be asked are (i) contribution of DcR gene inactivation in tumor regression to TRAIL

therapy, (ii) whether the combination TRAIL therapy sensitizes in cell lines stably depleted DcR expression is similar to constitutively inactivated lines, and (iii) effect of reconstitution of DcR expression on tumor regression *in vivo* to TRAIL-cisplatin treatment.

A robust *in vitro*and *in vivo* validation data demonstrating DcR inactivation on TRAIL-drug combination therapy will establish therapeutic efficacy superior to monotherapy and existing conventional therapies. This will establish decoy receptor inactivation as a biomarker of TRAIL therapy for CC and ultimately allow treatment stratification classes that were not possible so far.

METHODS

Tumor cell lines. Nine CC cell lines (C-4I, C-33A, HT-3, CaSki, ME-180, MS751, SW756, HeLa, and SiHa) from ATCC were used in previous studies will be used in these studies (Narayan et al., 2016).

Tissue culture, drugs and radiation treatment: Cells will be grown using standard conditions specific for each cell line and will be treated with defined concentrations and periods. The agonistic antibodies for TRAILR1 (mapatumumab) and TRAILR2 (lexatumumab) will be obtained from Human Genome Sciences. Cells will be exposed to gamma radiation using Gammacell 40 Cesium Unit (shared core facility at ICRC).

*CC cell line knockout and constitutive production of TNFRSF10C and TNFRSF10D***.** For *in vitro* experiments siRNA sequences of each gene will be commercially obtained along with negative controls. Transfections will be carried out, serially or simultaneously if two genes targeted, with Oligofectamine according to the manufacturer's protocol (Invitrogen). Efficiency of transfection will be judged either by RT-PCR or Western on cells collected after 48-72 hr. after transfection. Second transfection will be done if the reduction of RNA/protein is incomplete in the first transfection. Full-length ORF clones will be cloned in pcDNA and transfected along with vector controls by standard methods and stably selected clones will be used in both *in vitro* and *in vivo* testing of TRAIL-combination treatments using cytotoxicity and flow cytometry assays. For stable transfections, TNFRSF10C and TNFRSF10D pcDNA3.1 expression vectors will be generated (with and without luciferase labeling) by standard methods.The same genes will be knocked down using CRISPR/cas9 using standard methods. Stable cell lines generated by these transfections and transductions will be used for TRAIL-combination treatment experiments *in vitro* and *in vivo*.

Cytotoxicity and Flow cytometry: Cell viability and cytotoxicity against TRAIL and combination of other drugs will be assessed by standard colorimetric MTT and apoptosis by flow cytometry as described (Narayan et al 2011). All treatments will be performed in four replicate wells and in 3 repeats. LSR II flow cytometry instrument (BD Biosciences) is available as Shared Core facility at Columbia University.

Western blot analysis: Western blot analysis will be performed by standard methods. Immunoblotting will be performed using relevant antibodies.

Tumor xenograft and evaluation of drug response: All experiments will be conducted using protocols and conditions approved by the institutional animal care and use committee. Cell lines will be genetically modified to express *ffluc* gene by transfecting the vector containing the luciferase (Promega). Exponentially growing cells will be collected and resuspended in medium without serum at $1X10^8$ cells per ml. 10 million cells will be injected subcutaneously into dorsal flank or tail vein injection in NOD-SCID mice. Optimal dose of each drug will be determined by IC50 value for each cell line. For unmodified cell lines, in group one 10 mice will be injected for each cell line and a control group of 5 with vehicle. For the modified cell lines with 'knock-down' or 'knock-in' of specific DcR genes, 10 mice each will be injected for modified and parental cell line. Tumor growth will be monitored daily to allow 0.2-0.5 cm^2 on the longest axis before initiating the drug treatment. Alternatively, *in vivo* tumor growth will be monitored once a week by the luciferase activity by using bioluminescent image on anesthetized animals. The imaging will be performed at Confocal and Specialized Microscopy Shared Resource facility at Columbia University Cancer Center. Assessment of *in vivo* efficacy of TRAIL combination therapy of xenografts will be performed by tumor-derived bioluminescence using CCD camera (IVIS Spectrum System, Xenogen, Caliper Lifesciences) after injecting D-luciferin.Photons from the bioluminescence signal will be measured as total photon flux normalized to exposure time and surface areas and expressed in units of photon/second/cm^2/steradian. An estimated 6-8 imaging sessions for each animal for 10-12 weeks after injection is required. Animals will be monitored daily for normal behavior, weight loss, and other signs of clinical end points. The anti-tumor effect of drug therapy will be evaluated according to the sizes of tumors from 4 weeks and latter. Histological and cytotoxicity studies, when needed, will be performed on normal tissues and excised tumors by the Pathology core facility at Columbia University.

Statistical analysis: Standard statistical analyses will be performed with the help of biostatistics Shared Resource at Columbia University Medical Center and in designing the experiments that require statistical power.

SIGNIFICANT IMPACT OF RESEARCH IN FUTURE

The outcome of the proposed studies will provide a scientific rationale for selective use of combination agents that activate TRAIL pathway and a potential prognostic value in the prediction of TRAIL response, which will be a complete paradigm shift in CC treatment.

The present proposal seeks to further investigate this pathway to develop clinically significant therapeutic approach to stratify patients for TRAIL-combination therapy. Further investigate the molecular mechanisms regulated by *TNFRSF10C* and T*NFRSF10D*inactivation in TRAIL-mediated apoptosis in CC

These findings shed new light on the role of genetic/epigenetic defects in TRAIL decoy receptor genes in the pathogenesis of CC and provide an opportunity to explore strategies to test decoy receptor gene inactivation as a biomarker of response to Apo2L/TRAIL-combination therapy.

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