Tumor innervation in high-grade serous ovarian carcinoma

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Abstract

Patients with densely innervated tumors suffer a worse prognosis than those with sparsely innervated disease. Given the poor survival of ovarian cancer patients, we wondered if intra-tumoral neurons remain functional at the tumor bed and functionally contribute to disease progression. We analyzed ovarian cancers in The Cancer Genome Atlas (TCGA) for expression of neuronal genes. We used Proximity Ligation Assay (PLA) with pre-and post-synaptic markers to determine if synapse-like structures form at the tumor bed. We electrophysiologically analyzed HGSOC patient samples using Microelectrode Arrays (MEA) to determine if measurable activity is present in these malignancies. We also orthotopically implanted syngeneic HGSOC tumors into control and transgenic mice lacking tumor-infiltrating neurons and analyzed the electrophysiologic impact on tumors. Since we had previously defined tumor-infiltrating nerves as sensory, we tested the impact of Substance P on tumor cell proliferation and migration. Analysis of TCGA for neuronal genes shows that high neuronal gene expression correlated with low survival in high-grade serous ovarian carcinosm (HGSOC). PLA shows that cases of HGSOC harbor significantly higher PLA signal as compared to benign or normal ovary suggesting that intra-tumoral nerves form synapse-like structures in the malignancies. Consistent with this, MEA analysis shows significantly increased electrical activity in cases of HGSOC as compared to benign or normal tissues. Importantly, tumors grown in transgenic animals lacking tumor-infiltrating neurons harbor significantly decreased intra-tumoral electrical activity suggesting that at least some of this activity comes from intra-tumoral neurons. In vitro assays demonstrate that HGSOC cells respond to Substance P with increased proliferation and migration. These effects are blocked by inclusion of an NK1R antagonist. NK1R is the receptor for Subtance P and these studies indicate that the proliferative and migration impact of Substance P on HGSOC cells is mediated directly by binding of this neuropeptide to its receptor..HGSOCs are densely innervated by sensory nerves that remain functional at the tumor bed. Release of Substance P by these tumor-infiltrating nerves contributes to tumor cell proliferation and migration.

Introduction

The presences of intra-tumoral nerves is now widely accepted though their contributions to cancer initiation and progression remain largely undefined. We have previously published that most solid tumors are innervated to varying degrees. Importantly, patients with densely innervated tumors suffer a worse prognosis than those whose disease is sparsely innervated. The majority of patients with ovarian cancer get diagnosed at late stage which significantly contributes to their poor overall survival. Given the association of dense tumor innervation and poor prognosis, we wondered what if and how intra-tumoral nerves contribute to this poor outcome.

Here, we directly tested whether tumor-infiltrating nerves remain functional at the tumor bed. Moreover, we ask whether a neuropeptide released by tumor-infiltrating sensory nerves contributes directly to tumor growth and dissemination.

Methods:

Bioinformatic Analysis

We analyzed the expression of neuronal genes from The Cancer Genome Atlas (cbioportal.org) as well as the OncoLnc (www.oncolnc.org) databases to assess if the expression of these genes correlated with survival of patients with ovarian cancer.

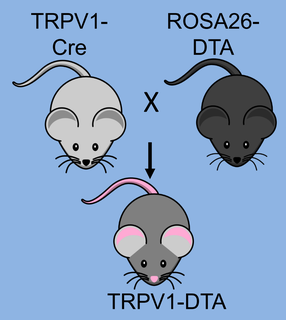
Proximity Ligation Assay

To assess whether tumor-infiltrating nerves form functional connections at the tumor bed, we used Proximity Ligation Assay (PLA). In this technique, two proteins of interest as assessed. Similar to immunofluorescent staining, antibodies to each protein are utilized to bind their targets. The secondary antibodies have small oligos attached to them. If the two proteins of interest are in close enough proximity to each other, these oligos can be ligated and then rolling circle amplification can be initiated. When complexed with a florophore, this results in a fluorescent spot over the region where the two proteins likely interact. If the two proteins are not in close enough proximity to each other, the oligos will not anneal and rolling circle amplification will fail and there will be no fluorescence. We performed PLA using the pre- and post-synaptic markers neuregulin and neuroligin-3 on n=5 samples of HGSOC, n=5 samples of normal ovary.

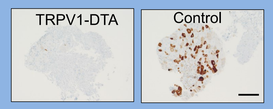
Microelectrode Arrays

Fresh cases of ovarian cancer were shipped overnight in transplant buffer from the BioTrust Collection at the University of Pennsylvania. Samples were immediately sliced, maintained in oxygenated artificial cerebrospinal fluid (to maintain neuronal health) and analyzed by MEA. MEAs contain multiple microelectrodes that stimulate and record electrical activity from overlying cells or tissue slices. Fresh tissue slices (n≥4 slices/patient sample depending on tumor size) were generated acutely from n= 13 HGSOC cases, n= 5 benign gynecological tumors and n=2 normal ovary. In addition to recording spontaneous electrical activity, MEA electrodes can be selectively stimulated and evoked responses from other electrodes then recorded. To ensure adequate contact between tumor slices and microelectrodes, we utilize perforated MEAs. The presence of a low-level vacuum generates negative pressure between the tissue slice and the perforations, ensuring that direct contact of tissue and electrodes is made which maximizes recording of electrical activity. The perforated MEA we utilize, pMEA100/30, contains a 6x10 electrode grid where 30 µm electrodes are spaced 100 µm apart.

In vivo studies



We crossed TRPV1-Cre and ROSA26-DTA (diphtheria toxin fragment A) mice to generate TRPV1-DTA animals. These progeny lack TRPV1 expressing cells, including neurons. We have previously published that ovarian cancers are innervated by TRPV1 expressing neurons. These double transgenic animals were validated by immunohistochemical staining for TRPV1 in dorsal root ganglia.



A syngeneic HGSOC cell line was used to orthotopically implant tumors into female control (C57Bl/6) and TRPV1-DTA animals. Tumors were allowed to grow and when sacrifice criteria were met, tumors were harvested and analyzed by MEA.

Western Blot Analysis

To determine the impact of Substance P (a major neuropeptide released by sensory nerves) on cellular proliferation, we first utilized western blot to determine whether ovarian cancer cells express the Substance P receptor, NK1R.

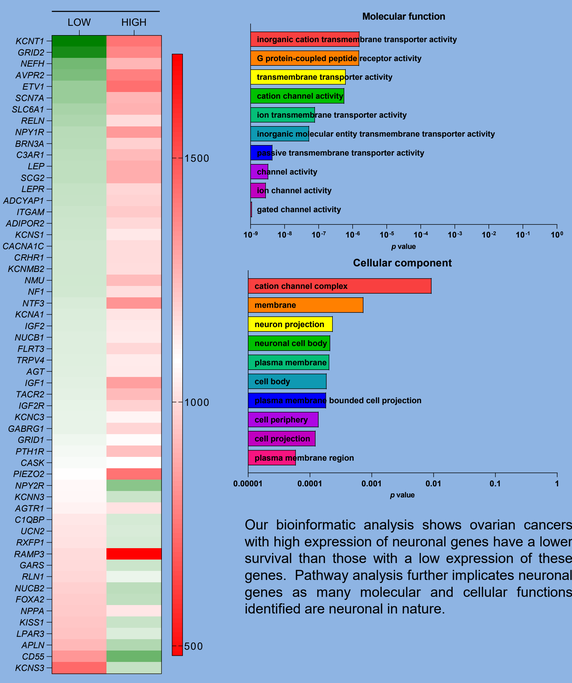
Proliferation assays

A syngeneic HGSOC cell line was used to test the impact of Substance P on cellular proliferation. Cells were treated with increasing concentrations of Substance P and cell numbers analyzed 24 and 48 hours later. To determine whether Substance P mediated its influence on cellular proliferation by binding to its receptor, NK1R, proliferation assays were repeated with inclusion of an NK1R antagonist.

Migration assays

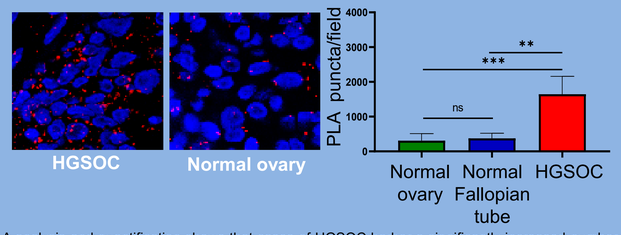
To assess the potential influence of Substance P on cellular migration, HGSOC cells were seeded on a transwell. Increasing concentrations of Substance P were placed in the bottom well and the number of cells migrating through the pores of the transwell were counted 24 and 48 hours later. To determine if proliferation was mediated by binding of Substance P to NK1R, the assays were repeated in the presence of an NK1R antagonist

Results

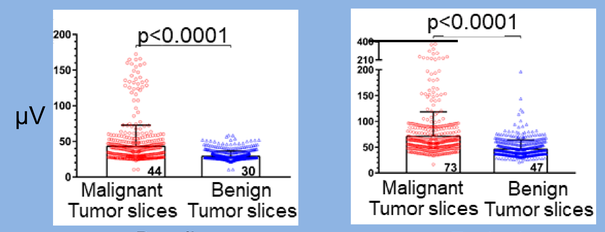


Our bioinformatic analysis shows ovarian cancers with high expression of neuronal genes have a lower survival than those with a low expression of these genes. Pathway analysis further implicates neuronal genes as many molecular and cellular functions identified are neuronal in nature.

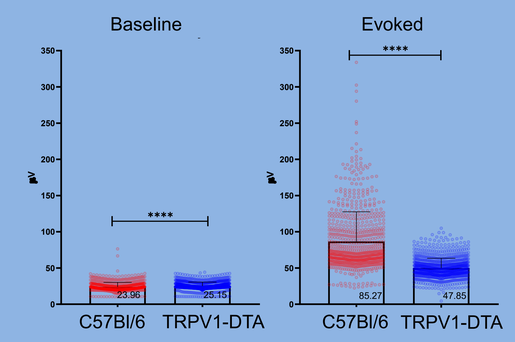
Results



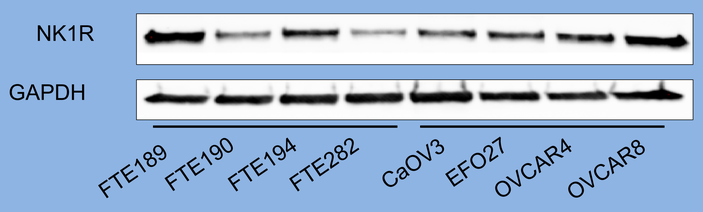
PLA analysis and quantification shows that cases of HGSOC harbor a significantly increased number of PLA positive puncta/field as compared to normal ovary and normal fallopian tube. These findings suggest that tumor-infiltrating nerves form synapse-like connections at the tumor bed.



Fresh cases of HGSOCs were analyzed by MEA. Electrical activity was recorded continuously. For all tissue slices, we utilized the same stimulating parameters as follows. Recordings of 1-5 minutes in duration/slice were performed. The electrical activity recorded from all electrodes for all samples was grouped and analyzed as follows: baseline activity was first recorded, after which selected electrodes are stimulated and evoked responses recorded. When the electrical stimulus is shut off, recordings continue to demonstrate reversion of electrical activity back to baseline. While the majority of samples harbored little to no spontaneous electrical activity; stimulation of one or more electrodes induced measurable evoked responses from other electrodes in all samples tested. Cases of HGSOC were compared to those of benign gynecologic disease (e.g. cystadenomas). The electrical activity measured from malignant tumor slices was significantly higher than that of benign disease. Shown above are graphs of baseline and evoked electrical activity of malignant and benign tumor slices.

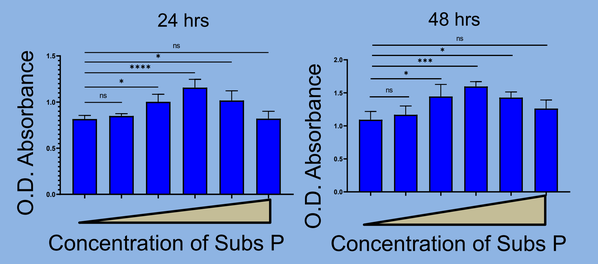


C57Bl/6 and TRPV1-DTA female mice were implanted with HGSOC tumors. When sacrifice criteria were met, animals were euthanized, tumors isolated and analyzed by MEA. Tumors grown in TRPV1-DTA animals had significantly reduced electrical activity suggesting that at least some of the electrical activity measured comes from tumor-infiltrating TRPV1-expressing neurons.

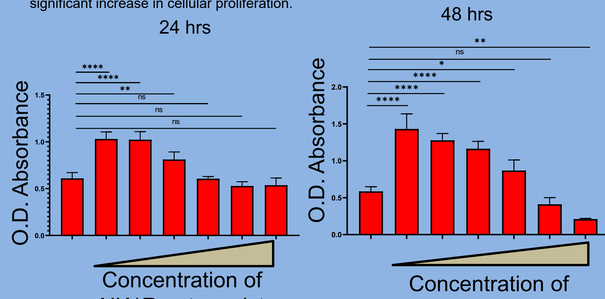


Western blot analysis of whole cell lysates of control (FTE) cell lines and four HGSOC cell lines (CaOV3, EF027, OVCAR4, OVCAR8) analyzed by western blot for expression of the Substance P receptor, NK1R. GAPDH was used as a loading control.

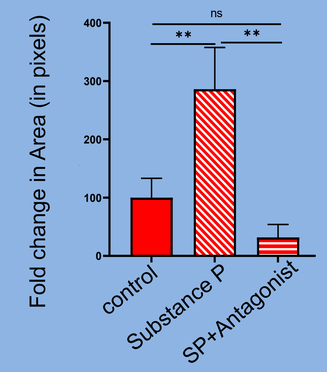
Results



In vitro proliferation assays of HGSOC cells treated with increasing concentrations of Substance P for 24 and 48 hours. HGSOC cells respond to substance P with a significant increase in cellular proliferation.



In vitro proliferation assays of HGSOC cells treated with of Substance P and an NK1R antagonist for 24 and 48 hours. These findings demonstrate that the response of HGSOC cells to Substance P is mediated by binding to NK1R.



In vitro migration (transwell) assay of HGSOC cells treated with substance P in the presence or absence of the NK1R antagonist.

Conclusions

- HGSOCs are highly innervated.
- HGSOCs that harbor high expression of neuronal genes have worse survival than those with low expression of neuronal genes.
- HGSOCs harbor elevated electrical activity.
- This electrical activity stems largely from TRPV1-expressing intra-tumoral neurons.
- Substance P promotes tumor cell proliferation.
- Substance P promotes tumor cell migration.

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