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Negative regulation of natural killer cell in tumor tissue and peripheral blood of oral squamous cell carcinoma

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ABSTRACT

Natural killer (NK) cells are the key lymphocytes in solid tumors. Its activity is regulated by both germline encoded receptors and cytokine microenvironment. We conducted a case-control study to investigate the activation status of NK cell in oral squamous cell carcinoma (OSCC). NK cell activation was assessed in context of NK cell cytotoxicity and transcript expression of NK cell receptors (NKp46 and KIRs) and NK cell associated cytokines (IL-1β, IL-2, IL-10, IL-12β, IL-15, IL-18, IL-21, IFN-γ, TNF-α and TGF-β). The results revealed possible mechanisms involved in reduced NK cell activation in peripheral circulation: quantitative deficiency of NK cell number and lowered cytotoxicity together with qualitative NK impairments caused by – (1) decreased expression of NK activating receptor NKp46, (2) increased expression of NK suppressive cytokines – IL-10 and TGF-β and (3) induction of $FOXP3^{+}CTLA4^{+}$ suppressor cells. On the other hand, in the tumor tissue, escape of NK immune surveillance appeared to be modulated by upregulation of TGF-β and IL-10 together with downregulation of NK cell activating cytokines (IL-2, IL-12β, IL-15, IL-18, IL-21 and IFN-γ) and NK receptors (NKp46 and KIRs). In addition, our study supported the earlier contention that TNF-α and IL-1β expression levels may be used as markers of malignant transformation in oral leukoplakia. In conclusion, the study provided an insight into the negative regulation of NK cell in tumor tissue and peripheral blood of OSCC patients, which can be exploited to boost the current NK cell and cytokine based immunotherapy for the treatment of oral cancer.

1. Introduction

Natural killer (NK) cells are the key immune lymphocytes in solid tumors [1]. They expresses many different receptors that can either trigger activation or mediate inhibition of their effector functions [2]. Receptors that mediate activation includes the Killer cell immunoglobulin-like receptors (KIRs), natural cytotoxicity receptors (NCRs), FcγRIIIA (CD16) and NKG2D (CD314). The KIRs, also referred to as CD158, are a group of polygenic and polymorphic family of receptors [3] expressed mainly in natural killer cells [4]. They are described as the key regulators for development, tolerance and activation of NK cells [5,6]. KIRs are encoded by a family of tightly clustered fourteen genes and two pseudogenes (2DP1 and 3DP1) on leukocyte receptor complex at chromosome 19q13.4 [7]. Of the 14 KIR genes, KIR3DL1-3 and KIR2DL1-5 are described as inhibitory KIRs, while KIR3DS1 and KIR2DS1-5 are activating KIRs [7,8]. These multigene KIRs interact with their polymorphic HLA -A, -B and -C ligands to diversify and individualize human immune system [9]. Apart from activating forms of KIRs (KIR2DS and KIR3DS), the other major lysis receptors in NK cell are the natural cytotoxicity receptors (NCRs). NCRs include three immunoglobulin-like proteins: NKp46 (NCR1; CD335), NKp44 (NCR2; CD336) and NKp30 (NCR3; CD337). Although NKp30 and NKp44 have similar cellular functions, NKp46 is structurally distinct from the other two molecules and is located in a different region of the genome [10,11]. It is stably expressed on both resting and activated NK cell, and is considered as a unique NK cell marker [10,11]. However, its ligand(s) remain elusive [12,13]. Recent

Abbreviations: OSCC, Oral Squamous Cell Carcinoma; AMCH, Assam Medical College and Hospital; GMCH, Gauhati Medical College and Hospital; NECHRI, North East Cancer Hospital and Research Institute; TUEC, Tezpur University Ethical Committee; PB, Peripheral blood.

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studies have demonstrated NKp46 to be critically involved in influenza [14,15], tumorogenesis [16] and diabetes [17,18]. It was described to be the key receptor in controlling the spread of various primary tumors in melanoma, lymphoma and carcinoma [12]. Further, experiments in an NKp46-knockout mouse model raised the possibility that the receptor is essential for controlling both cancer metastasis and influenza infection [12,14].

NK cell activity is not only regulated by its germline encoded receptors-ligand interaction but also by a correct cytokine microenvironment [19]. The prominent cytokines that are related to NK cell include the pro-inflammatory cytokines – Interleukin-1 (IL-1), IL-2, IL-12, IL-15, IL-18, IL-21, interferon-gamma (IFN-γ) and tumor necrosis factor-alpha (TNF-α), and anti-inflammatory cytokines – IL-10 and transforming growth factor-beta (TGF-β). Of these cytokines, IFN-γ, TNF-α, IL-10 and IL-1 are effector molecules of NK cell [20,21], while IL-2, IL-12, IL-15, IL-18 and IL-21 are NK cell-activating cytokines [2,19]. TGF-β and IL-10 are described as negative factors for NK cell activation [22–29]. The cytokines can influence NK cell effector functions either directly or by inducing other immune cells in the environment. The altered profiles of these cytokines have been implicated in pathogenesis of many diseases including cancer [19,30–34]. However, the nature of the relationship among these NK cell related cytokines, NK cells and tumor remains to be explored to full extent.

The present study aimed to investigate the role of NK cells in oral cancer, particularly NK cell activation in oral squamous cell carcinoma (OSCC). NK cell activation was assessed in the OSCC patients in context of – (1) NK cell cytotoxicity and (2) expression of NK receptors and key cytokines related to NK cell. Our data revealed that NK cell cytotoxicity in peripheral blood was lower in OSCC patients. Moreover, it was noted that the NK cells were negatively and differentially regulated in tumor tissue and in peripheral blood.

2. Materials and methods

2.1. Study design and participants

It was a hospital based case-control study conducted from April 2013 to June 2014. Clinically and histopathologically confirmed cases of OSCC were recruited from E.N.T. departments of three hospitals of Northeast India – Assam Medical College and Hospital (AMCH), Gauhati Medical College and Hospital (GMCH) and North East Cancer Hospital and Research Institute (NECHRI). The healthy participants were matched to cases by age (within 3 years), gender, ethnicity and geographic residence. The demographic, clinical and other characteristics of the patients and healthy control participants were recorded in questionnaires through personal interview, medical records and clinical examination by collaborating clinicians of the three respective hospitals. The tumor staging was done according to the American Joint Committee on Cancer (TNM) classification and grouped as early (clinical stages I–II) or advanced clinical stages (clinical stages III–IV) [35]. Histological grades of tumor were classified as – well differentiated, moderately differentiated or poorly differentiated as proposed by World Health Organization [36]. Institutional ethical committee – Tezpur University Ethical Committee (TUEC), Northeast India, approved the study. All individuals voluntarily agreed to participate and provided written informed consent.

2.2. NK cytotoxicity study

Peripheral blood mononuclear cells (PBMCs) was isolated from peripheral venous blood of 75 OSCC patients and 30 healthy participants by density gradient centrifugation using Histopaque (Sigma–Aldrich, Missouri, USA). NK cells were negatively separated from PBMCs using the human NK cell enrichment set – DM kit (BD Biosciences, New Jersey, USA) according to the manufacturer's instructions. NK cells were suspended in RPMI 1640 medium (Gibco, New York, USA) supplemented with fetal bovine serum, nonessential amino acids, sodium pyruvate, L-glutamine and antibiotic–antimycotic (Gibco) and used in cytotoxicity assays without delay. The erythroleukemic cell line K562 (Sigma–Aldrich) was used as target cells and were cultured in RPMI 1640 medium supplemented with foetal bovine serum and antibiotic–antimycotic.

The ability of freshly isolated peripheral blood NK (PB-NK) cell to lyse HLA-negative K562 target cells was measured by fluorescence microscopy using Live/Dead cell-mediated cytotoxicity kit (Molecular Probes, Oregon, USA). In the cell-mediated cytotoxicity kit, to distinguish K562 cells from NK cells, target cells were labelled with 3,3′-dioctadecyloxacarbocyanine ($DiOC_{18}$), a green fluorescent membrane stain. The labelled target cells were then incubated with the effector cells. After incubation, membrane-impermeant nucleic acid counterstain propidium iodide was added to label any cells with compromised plasma membranes. Both live and dead effector cells/target cells were readily discriminated in the fluorescence microscope. The dead target cells had coincident green-membrane and red-nucleus staining while dead effector cells had only red-nucleus staining.

The NK cell mediated cytotoxicity of K562 cells was calculated as cytotoxicity percentage (or lysis percentage against K562 cells) using the equation: (Dead K562 cells in presence of NK cells/Viable K562 cells in presence of NK cells) – (Spontaneous K562 cells death in absence of NK cells) × 100 or {(G + R cells/G cells) + effectors − (G + R cells/G cells) − effectors} × 100, where G = Green and R = Red.

2.3. Gene expression analysis

The expression of six groups of KIRs (2DL1, 2DL2 & 2DL3; 2DL5 A & B; 3DL1 & 3DS1; 3DL1; 2DS1, 2DS2 & 2DS4; 2DS3 & 2DS5), NKp46, NK cell associated cytokines (IL-1β, IL-2, IL-10, IL-12β, IL-15, IL-18, IL-21, IFN-γ, TNF-α and TGF-β), Forkhead Box P3 (FOXP3) and Cytotoxic T-Lymphocyte-Associated Antigen 4 (CTLA4) was measured in tissue and peripheral blood samples of 75 OSCC patients and 75 healthy participants by real-time quantitative reverse transcriptase PCR (qRT-PCR) using TaqMan gene expression assays (Applied Biosystems, California, USA) (Supplementary Tables 1–3). Briefly, total RNA was isolated from RNAlater stored blood and tissue samples of participants using RiboPure™ kit (Ambion Inc., Texas, USA) as described by the manufacturer. Yield (2–4 μg from 0.5 ml blood and 200–400 μg from 100 mg of tissue) and the purity (1.9–2.1) of the extracted total RNA were checked using Spectrophotometer. One microgram of total RNA was reverse-transcribed into cDNA using high-capacity cDNA reverse transcription Kit (Applied Biosystems) in a thermal cycler (Eppendorf, Hamburg, Germany) using random hexamers for initiating cDNA synthesis. The single-stranded cDNA products were then analyzed in real-time qRT-PCR.

Only samples with housekeeping genes (GAPDH and 18S rRNA) transcript levels within two standard deviations of the mean [37] were included for the expression study. 18S rRNA was used as the endogenous control for normalization of expression levels. The RNA transcript abundance for each gene was obtained by comparative cycle threshold (C_T) method [38].

2.4. Data analysis and statistical methods

Data analysis was done using statistical softwares – GraphPad Prism (La Jolla, CA, USA) and XLSTAT (Addinsoft, NY, USA). The