UNIVERSITY OF CALIFORNIA, SAN DIEGO

Regulation of NKG2D Ligand MICA by p53

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by

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The Thesis of Rod Seung-Hwan Lim is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

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ABSTRACT OF THE THESIS

Regulation of NKG2D Ligand MICA by p53

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NKG2D is a stimulatory receptor used by natural killer (NK) cells and some types of T cells of the immune system to detect infected and transformed cells. It recognizes and binds to NKG2D ligands—a family of ligands which are upregulated in diseased cells, which include MICA, MICB, and ULBP1-5 in humans and h60a-c, RAE-1a-e, and Mult-1 in mice. NKG2D ligands are expressed constitutively on primary tumors by poorly understood mechanisms. In addition, there is also active repression of NKG2D ligand expression by normal cells by mechanisms, which may be subverted in pathology by cancer cells and viruses. Therefore understanding the molecular mechanisms of upregulation during early stages of carcinogenesis and downregulation during later stages may have an important implication in cancer and immune disease therapeutics.

Introduction

Previously, we have shown that IFNs can downregulate the mouse NKG2D ligand H60 by decreasing H60 transcripts. In an effort to further characterize the mechanisms that regulate tumor immunity, we hypothesized that tumor suppressors, such as p53, in addition to using the intrinsic system by activating programmed cell death, may also use the extrinsic system by activating the immune system. Based on bioinformatics and published genome-wide chromatin immunoprecipitation (ChIP) data, which showed p53 occupation of the upstream regions of MICA, we hypothesized that p53 may regulate MICA. To test this hypothesis, we used Nutlin-3a, a small molecule which activates p53 by interrupting the interaction between p53 and its E3 ligase, MDM2. In all tested secondary fibroblasts and tumor cell lines with wildtype (WT) p53, we showed a profound increase in MICA transcript, but only a modest increase of surface expression of MICA; importantly, in cell lines that lack p53, no such change was observed. In addition, in cancer cell lines with WT p53 that showed a very small or no change in surface MICA protein, a large increase in soluble MICA could be detected in the supernatant. These results establish a role for p53 in regulation of immunogenicity. In addition, this new mechanism may explain the therapeutic effects of Nutlin, which is currently in Phase I clinical trial.

Chapter 1: General Introduction.

1.1 Role of p53 in tumorigenesis

Carcinogenesis is a phenomenon that involves a complex array of mutations giving rise to essential types of alterations in the genome commonly referred to as six hallmarks of cancer, which collectively determine the malignancy of tumor cells (Hanahan and Weinberg, 2000). It has been recently proposed that cancer immunosurveillance might be the seventh hallmark of cancer and various studies which examined animals that lack essential components of the immune system have supported this hypothesis (Bui and Schreiber, 2007; Dunn et al., 2004).

The transcription factor p53 is a tumor suppressor that responds to diverse stresses that participate in carcinogenesis. The gene encoding for the p53 protein and other components of the p53 pathway, its activator kinases and inhibitors MDM2 and MDMX, is mutated in virtually all cancers (Toledo and Wahl, 2006; Wahl et al., 2005). Despite decades of research in p53 function, its roles in regulating tumor immunity remain unclear.

1.2 The Regulation of NKG2D Ligands

One model for studying cancer immunosurveillance is the NKG2D and its ligands. NKG2D is a stimulatory immunoreceptor used by natural killer (NK) cells and some types of T cells of the immune system to detect infected and transformed cells. It recognizes and binds to NKG2D ligands—a family of ligands, which includes MICA, MICB, and ULBPs in humans and h60, RAE-1, and MULT1 in mice. NKG2D ligands are upregulated in tumors and they are induced by oncogenic signals, such as DNA damage and cellular senescence (Gasser et al., 2005;

Krizhanovsky et al., 2008). In addition, deficiency in NKG2D promotes development of spontaneous tumors in cancer models (Guerra et al., 2008).

Chapter 2: p53 Regulation of the NKG2D Ligand, MICA

2.1 Nutlin-3a upregulates MICA transcript and protein levels

To examine whether p53 regulates MICA, we first performed a bioinformatics search to examine the upstream regions of the MICA transcriptional start site and we were able to identify four canonical p53 response elements, each with two half sites, RRRCWWGYYY, around -10.5 kilobases from the MICA transcriptional start site (Fig. 1A). Interestingly, these were the same sites, which previous studies identified for increased p53 occupancy in response to DNA damage (Smeenk et al., 2008; Wei et al., 2006). We next asked whether p53 activation would lead to an increase in MICA mRNA levels, as predicted by the presence of p53 binding sites, by treating secondary human foreskin fibroblasts—CRL2076, CRL2096, and CRL2429, cancer cells with p53 function—HCT116 and U2OS, and cancer cells without p53 function—HCT116 $p53^{-/-}$, with Nutlin-3a. Consistent with the idea that MICA is a p53 target gene, we found a large increase in MICA mRNA after treatment with Nutlin-3a (24 hours, 10 μM), and the increase was not present in HCT116 $p53^{-/-}$. To control for off-target effects of Nutlin-3a, a vehicle control DMSO was used for all 'untreated' samples in the study.

NKG2D ligands function as transmembrane protein ligands for NKG2D. Therefore we next asked whether Nutlin-3a treatment results in an increase in surface expression of MICA by performing flow cytometry. We observed a modest increase in surface levels of MICA in CRL2096 and HCT116, but no such increase was observed in any of the cell lines lacking p53 function (Fig. 2A and B). Moreover, there was a trend toward increase in the BL2 cell line, which was statistically not significant (Fig. 2B). Time-course experiment showed that the increase in MICA protein was time-dependent, as shorter treatments resulted in smaller increases in MICA while longer treatments resulted in larger increases (Fig. 2C). To test whether the

Nutlin-3a treatment corresponded with general upregulation in immune ligands, we also analyzed the expression of HLA-A, B, and C, and did not find any statistically significant increase, although a trend toward increase was observed (data not shown). These data suggest that Nutlin-3a treatment results in increase in MICA surface expression in a p53 and cell-type dependent manner.

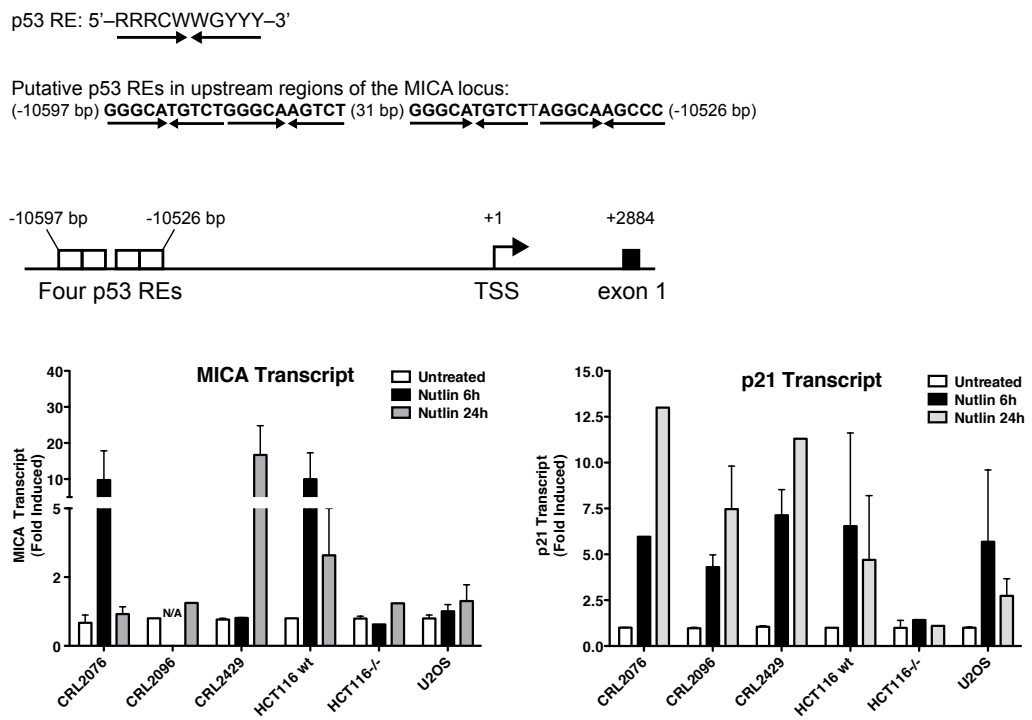

Figure 2.1. MICA contains p53 RE and is induced following Nutlin-3a treatment (A) Four canonical p53 response elements were present in the upstream regions, around -10.5 kb, of MICA transcriptional start site (TSS). The four putative response elements were closely clustered within 71 basepairs. R denotes purine, Y denotes pyrimidine, and W denotes A or T. (B) Nutlin-3a treatment strongly induced MICA mRNA in two human foreskin fibroblasts and HCT116 cell lines with p53. HCT116 $p53^{-/-}$ and other cell lines lacking p53 function did not show the same induction (data not shown). p21, a well-characterized target of p53, also showed a similar induction in response to Nutlin-3a. mRNAs are shown after normalization to GAPDH control, followed by another normalization to vehicle (DMSO) treatment condition.

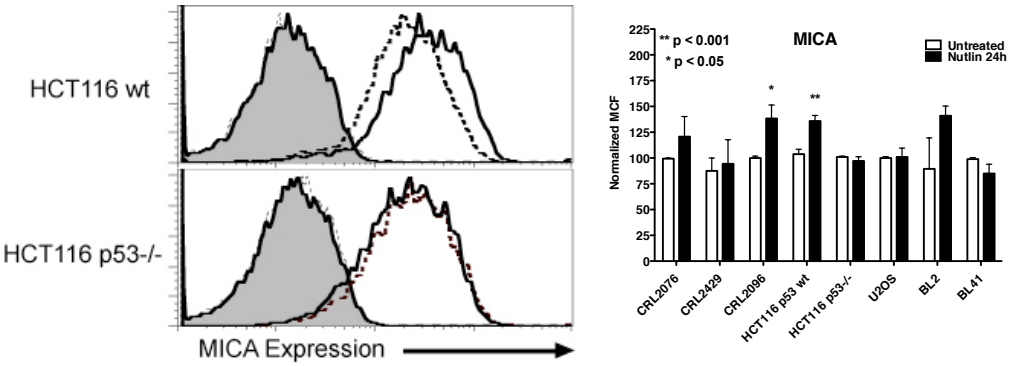

Figure 2.2. Nutlin-3a treatment induces surface expression of MICA. (A) Representative FACS histogram showing induction of MICA in HCT116 following Nutlin-3a treatment. No increase was observed in HCT116 $p53^{-/-}$. (B) Various cell lines were treated with 10 μM Nutlin-3a for 24 hours. Data are normalized to vehicle controls within cell lines and do not show the differences in expression levels of different cell lines. Only CRL2096 and HCT116 showed a statistically significant increase in MICA. (C) Time-course experiment with HCT116, treated with 10 μM Nutlin-3a and analyzed by FACS at five different time points.

2.2 Nutlin-3a increases soluble MICA

Although p53 activation resulted in increase in surface expression of MICA that was statistically significant in some cases, the increases were either nonexistent or modest in all cases. In comparison, the induction of MICA mRNA was much larger in magnitude. There are two non-exclusive explanations for this disparity in upregulation: some cells may have different post-transcriptional regulation of MICA or they may already be at physiological maximum threshold for MICA expression. Indeed tumor cells may possess both characteristics, as they have much higher basal expression of MICA and shedding of MICA by proteases has been observed both *in vitro* and *ex vivo* in cancer patient sera (Doubrovina et al., 2003; Groh et al., 2002a; Salih et al., 2002). Release of soluble MICA (sMICA) confers cancer cells with multiple benefits: initially, they reduce surface expression of MICA, thereby reducing direct recognition of cancer cells by immune cells with NKG2D (Doubrovina et al., 2003; Groh et al., 2002a; Salih et al., 2002). Moreover, chronic expression of sMICA is believed to have an immunosuppressive effect, as it leads to receptor downregulation (Groh et al., 2002a). Targeting sMICA is a potentially useful approach in cancer therapy, and it is currently under preclinical investigation

(Dranoff, 2008; Jinushi et al., 2006a). To assess whether there may be cell-type specific differences in post-transcriptional regulation of MICA, we assessed the sMICA levels in the supernatant by performing enzyme-linked immunosorbent assay (ELISA). Nutlin-3a treatment induced soluble MICA levels in three cell lines with p53 function (CRL2076, HCT116, and U2OS) but not in those without (HCT116 $p53^{-/-}$). Interestingly, magnitude of increase in sMICA in the two cancer cell lines was larger than what was observed in cells of non-tumor origin, despite the fact that the basal levels of sMICA was larger in cancer cells (data not shown). To test whether the increase in sMICA after Nutlin-3a treatment was due to an increase in cell death, we checked whether there was an increase in percentage of dead cells and observed no significant differences. Cells were also plated at near maximum densities to preclude the possibility that p53 activation results in cell numbers, thereby changing the soluble MICA levels.

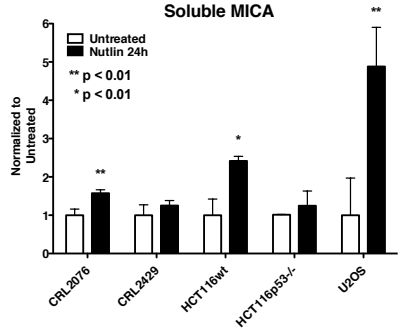

Figure 2.3. Nutlin-3a treatment increases soluble MICA levels in the supernatant. ELISA was performed following 10 μM Nutlin-3a treatment for 24 hours. CRL2429 and HCT116 $p53^{-/-}$ showed no increase in sMICA while CRL2076, HCT116, and U2OS showed modest to large increases.

2.3 Discussion

The NKG2D ligands and their activating receptor NKG2D play an important role in cancer immunosurveillance. NKG2D ligands are upregulated in response to environmental stress by poorly understood mechanisms. Here we show that the human NKG2D ligand MHC Class I-Related Chain A (MICA) is upregulated by activation of p53 by Nutlin-3a, a small molecule antagonist of mouse double minute-2 (MDM2) under preclinical investigations. Nutlin-3a treatment of tumor and secondary fibroblast cell lines resulted in p53-dependent induction of MICA transcript and protein. Furthermore, proteolytic shedding of MICA was strongly induced by p53 activation in tumor cell lines, whereas no such increase was observed for secondary fibroblasts. Thus, our data suggest that p53, which regulates cell-intrinsic mechanisms by inhibiting cell growth and activating programmed cell death, may also regulate cell-extrinsic mechanisms by activation of the immune system.

The data presented here indicate that MICA is induced by Nutlin-3a, and that it is a target of p53 activation. Nutlin-3a treatment of tumor and secondary fibroblast cell lines resulted in p53-dependent induction of MICA transcript and protein in a cell-type dependent manner. Moreover, p53 activation also induced shedding and release of sMICA in tumor cell lines. This suggests an intriguing possibility that tumor suppressors may induce immunogenicity of tumors and may participate in immunosurveillance.

Carcinogenesis is a phenomenon that involves a complex array of mutations giving rise to essential types of alterations in the genome commonly referred to as six hallmarks of cancer, which collectively determine the malignancy of tumor cells (Hanahan and Weinberg, 2000). It has been recently proposed that cancer immunosurveillance might be the seventh hallmark of cancer and various studies which examined animals that lack components of the immune system

have supported this hypothesis (Bui and Schreiber, 2007; Dunn et al., 2004). Recent *in vivo* studies that examined the role of p53 activation also suggest that it may play a role in regulating immunogenicity, in addition to inhibiting growth and triggering apoptosis (Xue et al., 2007). In addition, *in vitro* studies have also suggested a role of senescence and p53 regulating immunogenicity, by inducing ligands that activate NK cells (Krizhanovsky et al., 2008). It is possible that p53 may also regulate other NKG2D ligands, as MICB and ULBP2 also have p53 response elements in the upstream regions. Indeed, consistent with others findings, our data suggest that ULBP2 might be induced by p53 or senescence (Supplementary fig.; (Krizhanovsky et al., 2008). Nevertheless, the regulation may be complex, as p53 is a pleiotropic effector and it has been known to regulate microRNAs, some of which also regulate NKG2D ligands (He et al., 2007) . Further studies are needed to elucidate the post-transcriptional regulation of NKG2D ligands following p53 activation, as they may account for the differences between cell types in MICA expression.

It is also interesting to note that, in the mouse, p53 was observed to be dispensable for upregulation of NKG2D ligands following DNA damage (Gasser et al., 2005). Our findings also suggest that p53 may not be necessary for MICA expression, as HCT116 $p53^{-/-}$ cells do not have much lower expression of MICA than its wild-type counterpart. It is therefore likely that p53 is one of many regulators of NKG2D ligand transcription and indeed disparate signaling pathways have been implicated in NKG2D ligand induction. Alternatively, it is possible that in the absence of p53, the p53 binding sites may be acted upon by p63 and p73, which have been observed to bind p53 DNA target sites and transactivate p53-target genes.

Chapter 3: Materials and Methods

3.1 Cell Culture and Reagents

CRL-2076, CRL-2096, and CRL-2429, human osteosarcoma U2OS, and HCT116 cells were purchased from American Type Culture Collection (Manassas, VA). HCT116 $p53^{-/-}$ cells were kindly provided by Dr. B Vogelstein (Baltimore, MD). Human Burkitt lymphoma cells, BL2, BL40, and BL41, were kind gifts from Dr. Martin Allday (London, UK). Human osteosarcoma U2OS cells were purchased from ATCC and CRL-2076, CRL-2096, CRL-2429, BL-2, BL40, and BL-41 cells were cultured in RPMI/10% heat-inactivated fetal bovine serum with penicillin/streptomycin. HCT116 cells were cultured in McCoy 5A medium supplemented with 10% FBS (100 μg/mL) and penicillin (100 units/mL). U2OS cells were cultured in DMEM supplemented with 10% fetal bovine serum (FBS). Nutlin-3a (Dr. L. Vassilev, Hoffman-La Roche) was used at 10 μM for 24 hours unless otherwise noted; Nutlin-3 purchased from Cayman Chemical was also tested.

3.2 Flow Cytometry

Cells were harvested without trypsin and stained with Abs to MICA, MICB, ULBP1–3 (R&D Systems), HLA-ABC-FITC, or appropriate isotype controls (eBioscience). Secondary Abs used were either goat anti-mouse IgG-APC (eBioscience) or rat anti-mouse IgG2a/b-PE (BD Biosciences).

3.3 Quantitative RT-PCR

MICA and miRNA Taqman primers and cDNA synthesis kits were purchased through Applied Biosystems. p21 Taqman primer was used as previously described (Stommel and Wahl, 2004). Total RNA was extracted using RNABee (Tel-Test, Friendswood, Texas). RT reactions were normalized to GAPDH for MICA and p21 transcripts.

3.4 ELISA

The sandwich ELISA for measuring soluble MICA (sMICA) was performed according to the manufacturer's protocol (DuoSet ELISA Development System MICA ELISA kit, R&D Systems, Inc., Minneapolis, MN).

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