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Research paper

Anterior Gradient-3: A novel biomarker for ovarian cancer that mediates cisplatin resistance in xenograft models

Terry A. Gray [a], Nicola J. MacLaine [a], Caroline O. Michie [c], Pavla Bouchalova [d], Euan Murray [a], Jacqueline Howie [a], Roman Hrstka [d], Magdalena M. Maslon [a], Rudolf Nenutil [d,e], Borek Vojtesek [d], Simon Langdon [b], Larry Hayward [b], Charlie Gourley [c], Ted R. Hupp [a,*]

[a] *p53 Signal Transduction Group, Edinburgh Cancer Research Centre, Institute of Genetics and Molecular Medicine, University of Edinburgh, Crewe Road South, Edinburgh, EH4 2XR, Scotland, UK*
[b] *Edinburgh Breakthrough Research Unit and Division of Pathology, Edinburgh Cancer Research Centre, Institute of Genetics and Molecular Medicine, University of Edinburgh, Crewe Road South, Edinburgh, EH4 2XR, Scotland, UK*
[c] *Ovarian Cancer Genetics and Translational Research, Edinburgh Cancer Research Centre, Institute of Genetics and Molecular Medicine, University of Edinburgh, Crewe Road South, Edinburgh, EH4 2XR, Scotland, UK*
[d] *Regional Centre for Applied Molecular Oncology, Masaryk Memorial Cancer Institute, 656 53 Brno, Czech Republic*
[e] *Department of Oncological and Experimental Pathology, Masaryk Memorial Cancer Institute, 656 53 Brno, Czech Republic*

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ABSTRACT

The Anterior Gradient (AGR) genes *AGR2* and *AGR3* are part of the Protein Disulfide Isomerase (PDI) family and harbour core thioredoxin folds (CxxS motifs) that have the potential to regulate protein folding and maturation. A number of proteomics and transcriptomics screens in the fields of limb regeneration, cancer cell metastasis, pro-oncogenic oestrogen-signalling, and p53 regulation have identified AGR2 as a novel component of these signalling pathways. Curiously, despite the fact that the *AGR2* and *AGR3* genes are contiguous on chromosome 7p21.1-3, the AGR3 protein has rarely been identified in such OMICs screens along with AGR2 protein. Therefore there is little information on how AGR3 protein is expressed in normal and diseased states. A panel of three monoclonal antibodies was generated towards AGR3 protein for identifying novel clinical models that can be used to define whether AGR3 protein could play a positive or negative role in human cancer development. One monoclonal antibody was AGR3-specific and bound a linear epitope that could be defined using both pep-scan and phage-peptide library screening. Using this monoclonal antibody, endogenous AGR3 protein expression was shown to be cytosolic in four human ovarian cancer subtypes; serous, endometrioid, clear cell, and mucinous. Mucinous ovarian cancers produced the highest number of AGR3 positive cells. AGR3 expression is coupled to AGR2 expression only in mucinous ovarian cancers, whereas AGR3 and AGR2 expressions are uncoupled in the other three types of ovarian cancer. AGR3 expression in ovarian cancer is independent of oestrogen-receptor expression, which is distinct from the oestrogen-receptor dependent expression of AGR3 in breast cancers. Isogenic cancer cell models were created that over-express AGR3 and these demonstrated that AGR3 mediates cisplatin-resistance in mouse xenografts. These data indicate that AGR3 is over-expressed by a hormone (oestrogen-receptor α)-independent mechanism and identify a novel protein-folding associated pathway that could mediate resistance to DNA-damaging agents in human cancers.

1. Introduction

Anterior Gradient-2 (AGR2) was originally identified as a gene expressed in *Xenopus* development that specifies dorso-anterior ectodermal fate during the formation of the

* Corresponding author. Tel.: +44 1317773542; fax: +44 131777358.
E-mail address: ted.hupp@ed.ac.uk (T.R. Hupp).

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cement gland, a mucus-secreting organ involved in the attachment of the embryo to a solid support prior to swimming and feeding (Sive et al., 1989; Sive and Bradley, 1996; Aberger et al., 1998). *AGR2* is now known to have an orthologue named *AGR3* which together are part of the Protein Disulfide Isomerase (PDI) family (Persson et al., 2005). There are at least 18 human PDI family members (Hatahet and Ruddock, 2009) (Appenzeller-Herzog and Ellgaard, 2008). Protein Disulfide Isomerases (PDIs) contain core thioredoxin folds (CxxC or CxxS motifs), which regulate protein folding via regulation of disulfide bond formation (Persson et al., 2005). There are five highly conserved members within the AGR2 grouping that include: TRX1 (thought to be predominantly the nuclear thioredoxin), TRX2 (thought to be predominantly the mitochondrial thioredoxin), endoplasmic reticulum (ER) protein 18 (ERP18 (Alanen et al., 2003) which is the ancestral founder in the AGR2/3 group), AGR2, and the AGR2 orthologue AGR3. AGR2 and AGR3 are confined to vertebrates and both have the CxxS core motif instead of the classic thioredoxin fold/CxxC motif of TRX1, TRX2, and ERP18.

AGR2 is now known to be involved in normal intestinal mucus production (Park et al., 2009) and in normal limb regeneration in the newt (Kumar et al., 2007). AGR2 expression is also perturbed in human disease; it is part of a stress protein group that can be used as a diagnostic tool for oesophageal disease (Barrett's) (Groome et al., 2008) and in the aetiology of asthma and inflammatory bowel disease (Zheng et al., 2006; Di Valentin et al., 2009). Transcriptomic and proteomic approaches in the past ten years using human tissue biopsies have identified novel, clinically-relevant protein families involved in cancer that have not been identified from cancer genetic screens. AGR2 forms a prototype case as a gene not identified from powerful cancer genetics and oncogene discovery models of the 1980s and 1990s, but from more latterly developed OMICS expression screens that reflect dynamic transcriptional and translational functions of a cell that drive oncogenesis. AGR2 is involved in tumour-associated pathways, including tumour growth, cellular transformation, cell migration, metastasis and chemoresistance (Liu et al., 2005; Ramachandran et al., 2008; Wang et al., 2008), and may represent a novel prognostic factor in some cancer types (Smirnov et al., 2005; Barraclough et al., 2009). Human AGR2 can be induced by the oestrogen receptor α (ERα) (Thompson and Weigel, 1998) and clinical studies suggested a positive correlation between AGR2 levels and ERα expression (Fletcher et al., 2003). Surprisingly however, AGR2 expression is induced rather than suppressed by the anti-oestrogen cancer drug Tamoxifen thus identifying AGR2 as an important pro-oncogenic agonist of Tamoxifen (Hrstka et al., 2010). A label-free data-independent proteomics screen (PACIFIC) of Tamoxifen-treated MCF7 cancer cells identified AGR2 protein as one the top proteins elevated in Tamoxifen treated cells (Hengel et al., accepted for publication). Clinical studies have also shown that AGR2 expression can also predict poor responses to Tamoxifen in breast cancer patients identifying a potentially novel pathway to target for treating drug resistant cancers (Hrstka et al., 2010).

Oestrogen-independent expression of AGR2 can be observed in other human cancers including prostate (Fletcher et al., 2003; Kristiansen et al., 2005; Zhang et al., 2005, 2007), lung (Fritzsche et al., 2007; Zhu et al., 2007), pancreas (Missiaglia et al., 2004; Lowe et al., 2007; Ramachandran et al., 2008), liver (Lepreux et al., 2011), and oesophagus (Iacobuzio-Donahue et al., 2003; Hao et al., 2006). An oesophageal cancer progression model has also identified a function for AGR2. Oesophageal adenocarcinoma similarly proceeds by a "stepwise" process including mutation of p53 and p16 that drives the development of metaplasia, dysplasia, and adenocarcinoma (Leedham et al., 2008). Oesophageal adenocarcinoma differs from other cancer types in that the environmental stress of bile acid reflux plays an apparently important role in disease progression and that p53 tumour suppressor gene mutation can occur very early in the carcinogenic sequence (Leedham et al., 2008). The early selection pressure for p53 mutation in oesophageal cancer occurs during the replacement of squamous epithelium with metaplastic epithelium also called "Barrett's oesophagus". In order to identify novel pro-oncogenic proteins that function as proto-oncogenes in Barrett's epithelium, a proteomics screen was initiated to identify candidate proteins over-produced in Barrett's compared to normal squamous epithelium. This screen identified a highly over-expressed protein named Anterior Gradient-2 (AGR2) that was shown to inhibit the p53 response to DNA damage thus identifying a clinically relevant, novel p53 silencing pathway in metaplasia (Hopwood et al., 1997; Yagui-Beltran et al., 2001; Pohler et al., 2004). AGR2 mRNA expression is also a dominant feature of a recently identified murine model of Barrett's oesophageal epithelium induced through deletion of the squamous stem cell progenitor p63 (Wang et al., 2011). AGR2 is now known to be over-produced in the majority of oesophageal squamous cancers suggesting that once it is elevated in Barrett's metaplasia, selection pressures are placed on maintaining its expression (Dong et al., 2011).

It is notable that AGR2, and not AGR3, is the gene/protein that was found to be expressed by oestrogen (Thompson and Weigel, 1998), as a dominant protein induced in Tamoxifen treated cells (Hengel et al., accepted for publication), as an abundantly expressed p53-inhibitor in a Barrett's oesophagus proteomic screens (Pohler et al., 2004) (Murray et al., 2007), and as a predictive marker for Letrozole responses in patients (Mackay et al., 2007). As such, there are no molecular studies to indicate whether AGR3, like AGR2, is pro-oncogenic, anti-oncogenic, or perhaps functions in a redundant manner with AGR2. AGR3, also known as HAG-3, AG3 or BCMP11, was first identified using a proteomics screen as a protein present in breast cancer cell membrane fractions (Adam et al., 2003). Follow-up studies confirmed that AGR3 is over-expressed in breast tumours and that AGR3 is co-expressed with AGR2 in breast cancer tissue with a strong positive correlation with ERα status (Fletcher et al., 2003). However, AGR3 was not shown to be co-expressed to a high degree with AGR2 in prostate cancers, indicating that AGR2 and AGR3 expressions can be uncoupled. As there has been limited data published on AGR3 and considering the growing amount information on AGR2 functions in health and disease, we investigated the expression of AGR3 in human cancer in order to identify a suitable clinical model in which to begin to study AGR3 function. We therefore set out to generate immunochemical tools and to identify models in which to study the putative role of AGR3 in human cancer development. We have developed a panel of