Journal of
Clinical & Cellular Immunology

Barden et al., J Clin Cell Immunol 2014, 5:4
DOI: 10.4172/2155-9899.1000237

Research Article Open Access

Non-Functional $P2X_7$: A Novel and Ubiquitous Target in Human Cancer

Julian A. Barden[1*], Aysen Yuksel[1], John Pedersen[2], Suzanne Danieletto[3] and Warick Delprado[4]

[1]*Biosceptre International Ltd, Riverside Life Sciences, 11 Julius Ave, North Ryde, N.S.W. 2113, Australia*

[2]*TissuPath, 96B Ricketts Road, Mt Waverley Vic 3149, Australia*

[3]*SAN Pathology Department, 185 Fox Valley Rd, Wahroonga, N.S.W 2076, Australia*

[4]*Douglass Hanly Moir Pathology, 14 Giffnock Ave., Macquarie Park, N.S.W. 2113, Australia*

[*]**Corresponding author:** Dr. Julian A. Barden, Director of Research, Biosceptre International Ltd, 11 Julius Ave, North Ryde NSW 2113 Australia, Tel: + 61 2 9490 8280, + 61 419 998 918; Fax: + 61 2 9490 5038; E-mail: Julian.barden@biosceptre.com

Received date: June 05, 2014, **Accepted date:** July 11, 2014, **Published date:** July 18, 2014

Abstract

The World Health Organisation reports there were 14.1 million new cancer cases and 8.2 million cancer deaths in 2012 demonstrating significant unmet medical needs exist. Modifications to cell death mechanisms that take place during cancer and the significant proliferative and metastatic potential of cancer cells have focused medical research onto targets and pathways that are known to control these critical functions. One such target is the $P2X_7$ receptor. $P2X_7$ is a purinergic receptor that forms ATP-gated channels ultimately mediating proliferation or cell death, depending on activation conditions. Recent preclinical studies have investigated the possibility that modulators of the $P2X_7$ channel may provide innovative cancer therapeutic approaches. The $P2X_7$ receptors expressed on cancer cells are found in a non-functional conformation we define as $nfP2X_7$. The $nfP2X_7$ receptor is unable to form the large pore conformation of the channel that is associated with driving cell death. In the present study we utilise antibodies that are specific to the $nfP2X_7$ form of $P2X_7$ to confirm the expression of $nfP2X_7$ receptor in a panel of human cancer tissues. We demonstrate that the $nfP2X_7$ is expressed ubiquitously on the surface of cancer cells and thus has potential to provide a novel and broad therapeutic cancer target.

Keywords: Cancer; $P2X_7$; Tumor

Introduction

Cancer in humans constitutes a set of about a hundred different diseases characterised by the uncontrolled growth of the affected cells often combined with the ability to spread, through metastasis, to distant sites. In examining this set of common characteristics, one is struck by the possibility that the inability of the affected cells to die at their allotted time may suggest that a common factor or master mechanism is present. The search for one or more elusive magic bullets as proposed by the Nobel Laureate Paul Ehrlich and others over a century ago [1] was always predicated on the hope of identifying a common target or pathway. Such a discovery would enable the apparent innate complexity in cancer to be greatly simplified. The current consensus view is that widely observed cancer cell population heterogeneity makes the targeting either of individual mutations on cells or single signalling pathways too limited for such approaches to be fully curative.

The current complexity is best illustrated by a recent report [2] in which the authors highlighted the prodigious effort expended on developing and testing treatments for the multitude of different human cancers for which there remains an enormous unmet need. A total of 981 were either in clinical trial or under review by the US FDA. Of the 84 actively sought cancer targets in current development, the top 8 are the focus of about 235 of the drugs that are now in advanced clinical development. These include VEGFR, PI3K, HER2, EGFR, PDGFR, c-Kit, cMet and mTor. Targeting of up-regulated receptors or pathway components present on normal cells is sub-optimal given the propensity for such procedures to generate unwanted side effects. The search for a genuine target specific for cancer cells is, therefore, vitally important, whether it is confined to a single tissue sub-indication or is present on a wide variety of cancer types. Specificity for the target is paramount, both for avoiding unwanted side effects and for minimising the required therapeutic dose that is otherwise lost through unwanted binding to normal (non-target) tissue.

It should not be surprising that in searching for a common target, an investigation was made of events central to the failure of cancer cells to die on schedule via normal activation of apoptosis. One potential common target receptor, $P2X_7$, is involved both in proliferation and in apoptosis. $P2X_7$ is a purinergic receptor that forms an ATP-gated cation-selective channel mediating cell death in haematopoietic and immune cells such as thymocytes [3], dendritic cells [4], lymphocytes [5,6], macrophages [7] and monocytes [8]. $P2X_7$ is also expressed on other cell types such as those possessing epithelial, mesenchymal and neural lineages [9-12]. These receptors have two transmembrane domains separating an extracellular domain that exhibits extensive homology with other P2X subtypes. $P2X_7$ has a short N-terminal intracellular segment and, unlike other P2X subtypes, a long intracellular C-terminal domain [13] that confers pore-forming properties on the homotrimeric, assembled channel in the plasma membrane [14]. Prolonged exposure of the assembled channel to ATP results in additional pore dilation [15]. A rapid influx of calcium ensues, activating various proteases, such as caspases [16], leading to apoptosis or programmed cell death [17].

Over 80% of human tumours arise from epithelial cells, most frequently at mucosal surfaces. Early studies provided preliminary evidence that the onset of cancer in these tissues is universally accompanied by $P2X_7$ receptor expression [9-12]. Importantly, these cancer cell-expressed receptors are deployed in a non-functional

conformation [18], rendering them unable to form apoptotic pores either through an inability to bind ATP to each of the separate sites on the receptor or due to the inability of all three monomers to pack appropriately in the cell membrane [19]. They are therefore unable to initiate apoptosis, preventing cells expressing this receptor from undergoing programmed cell death. However, while unable to form an apoptotic pore, the deployed receptors on cancer cells, referred to as $nfP2X_7$, maintain residual calcium signalling activity as a result of one of the ATP binding sites maintaining integrity [20]. Thus, not only is $nfP2X_7$ expressed in increasing amounts in a failed attempt to clear the aberrant cell, but the effect of an unwanted residual non-selective Ca^{2+}, Na^+ and K^+ channel on the plasma membrane of these cells serves to eventually provide them with their undesirable metastatic potential [20]. Residual channel activity leads to morphological changes including membrane blebbing [17], a general rounding up of the cell [21] and the shedding of various anchorage or adhesion proteins otherwise capable of holding the cell within the primary tissue. These include L-selectin, matrix metalloproteinase [15] and cathepsins [22-24]. The most metastatic cells, those with the highest invasion potential, appear to express more of the aberrant $nfP2X_7$ on their surfaces.

The inability to undergo apoptotic pore formation once the receptors are up-regulated on the target cell surfaces could result from rare splice isoforms or single nucleotide polymorphisms [25]. Alternatively, one of the monomers in the assembled trimer may be improperly packed due to problems with one or more of the eleven intracellular accessory binding proteins. Irrespective of the causes, two of the three ATP binding sites on the assembled receptor, formed only through correct packing of the monomers at the intermonomer interface, are exposed. This leaves just one site that is assembled and functioning correctly to provide the measured residual channel activity. The loss of the remaining two ATP sites through faulty packing of one of the monomers prevents the binding of all three ATP molecules required to activate pore formation. This provides a selective target on $nfP2X_7$ that is unavailable on $P2X_7$ [19,26-28]. Selective targeting of an otherwise hidden epitope allows for exquisite discrimination betweennormal cells and those expressing non-functional receptors.

Here we initially catalogue the expression of $nfP2X_7$ receptor in a panel of human cancer tissues and show this receptor is expressed ubiquitously on the surface of cancer cells and thus has potential as an entirely novel and broad therapeutic target.

Methods

Specimens

Tissues were obtained in large microarrays from Oncotest GmbH (Freiburg) and the laboratories of contributing authors.

Tissue and cell labelling

Tissue sections (5 µm) were de-waxed and heat treated at 97°C for 50 minutes in Universal Decloaker solution (Biocare Medical), rinsed in TBS Auto wash buffer (Biocare Medical) and mounted in a Sequenza slide stainer (Thermo Electron Corp.). Sections were labelled with the GMP-produced primary mouse monoclonal antibody of type IgG2aλ designated BPM09.1-002 [29] at a concentration of 500 ng/mL in Da Vinci Green Antibody Diluent (Biocare Medical) for 1h at room temp and rinsed twice in TBS Auto wash buffer. Slides were then treated with the Mach 4 universal detection system (Biocare Medical) consisting of 15 min in Mouse Probe followed by a rinse in TBS, then 25 min in HRP Polymer followed by two rinses in TBS. Slides were incubated with DAB (Biocare Medical) for 5 minutes, rinsed for 10 min in TBS and counterstained by a brief dip in Meyer's Hematoxylin, Lillie's Modification (*Dako*, Carpintera, CA), rinsed for 5 min in TBS, briefly rinsed in water, air dried and mounted in Entellan mounting medium (Merck). Slides were photographed using a QImaging Micropublisher 5.0 RTV camera mounted on an Olympus BX41 microscope using 20x objective. Slides were stored in a slide cabinet. All normal control sections were negative for $nfP2X_7$.

Results

Target receptor distribution was investigated through examining a wide selection of cancer tissue types. Within several of the major indications, a large number of individual cases were examined to establish how ubiquitous the marker appears to be in the general population. In excess of 100 different cases of prostate, skin and breast cancers were examined. Figure 1 shows representative examples from normal prostate (Figure 1a), normal breast (Figure 1c) and normal skin (Figure 1e) versus corresponding examples of cancer tissue including prostate cancer (Figure 1b), breast cancer (Figure 1d) and melanoma (Figure 1f) all stained with a monoclonal antibody specific for $nfP2X_7$ and adapted for use in paraffin-embedded fixed tissue samples most easily accessible in tissue banks. Similarly, cancer cell lines derived from these tissues were found to express $nfP2X_7$. Examples shown include prostate PC3 cells (Figure 1g) and breast MCF7 (Figure 1h).

As described [9-11], in the most advanced stages of the cancer, the $nfP2X_7$ receptors tend to be found on the plasma membrane with correspondingly little residual cytoplasmic receptor. In contrast, lower grade tumours exhibit a preponderance of cells in which a large proportion of the receptors remain intracellular. Such a progressive transport of non-functioning apoptotic receptors to the plasma membrane as disease progresses predicts that cancer cell lines should exhibit $nfP2X_7$ receptors on the plasma membrane rather than being largely intracellular. Figures 1g and 1h confirm that the receptors appear in their terminal deployment on the plasma membrane. These cells were non-permeabilised so only the epitope located in the extracellular domain of the receptor on the plasma membrane was labelled. The confocal images show that no stain penetrated the plasma membrane.

Several other epithelial cell cancers are shown in Figure 2. They include bowel adenocarcinoma (Figure 2a), ovarian serous tumour (Figure 2b), cervical cancer (Figure 2c), endometrial carcinoma of the uterus (Figure 2d), small cell lung cancer (Figure 2e), hepatocellular carcinoma (Figure 2f), transitional cell carcinoma of the bladder (Figure 2g) and Barrett's mucosa with adenocarcinoma (Figure 2h). These tissues were sampled in large numbers (n=20-100). All epithelial cancer samples examined express $nfP2X_7$.

Various breast cancer pathologies, including both invasive and *in situ* lobular and ductal carcinomas were also examined for $nfP2X_7$. All types expressed non-functional receptors. In contrast, normal breast epithelial tissue and numerous other normal tissues such as bowel, bladder, ovarian, uterine, cervical, stomach and lung were found to be devoid of $nfP2X_7$. However, areas of apparently morphologically normal tissue surrounding tumours in prostate also expressed the receptors. This is in line with earlier reports of the field-effect in which

tumour cells secrete growth factors that permeate surrounding normal epithelium in connected ducts thereby alerting these cells to the presence of an adjacent developing tumour. Initially, these cells begin deploying receptor in an entirely intracellular location but eventually they are deployed on the plasma membrane as well as on the basement membrane prior to stromal invasion. While all cancer tissues examined expressed $nfP2X_7$, no normal epithelial tissue displayed plasma membrane $nfP2X_7$ [11].

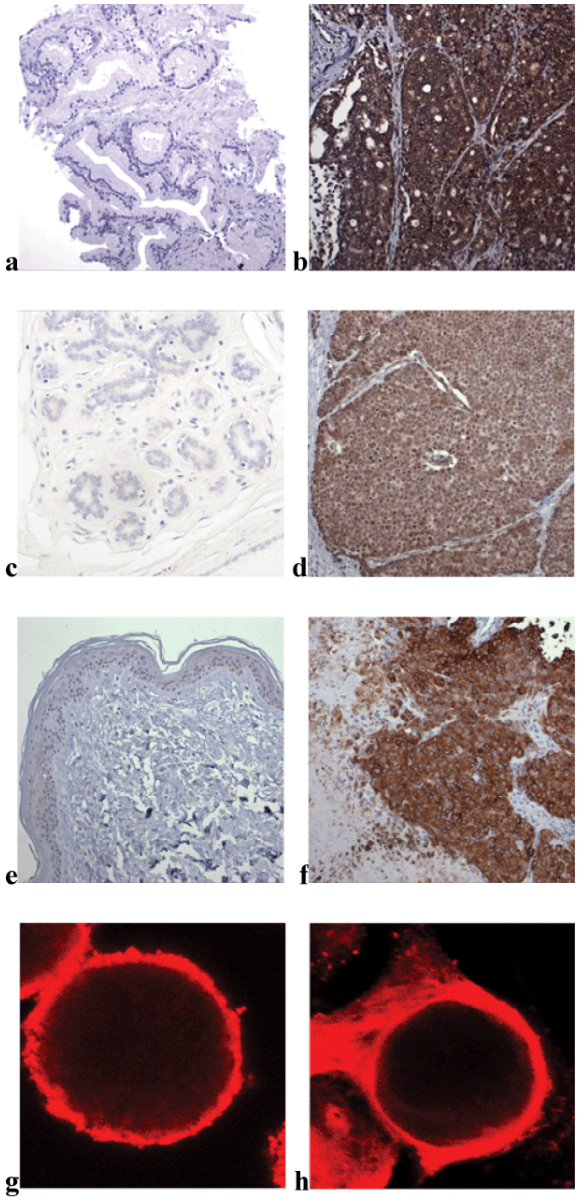

Figure 1: Sections of: **(a)** normal prostate; **(b)** prostate cancer; **(c)** normal breast; **(d)** invasive breast cancer; **(e)** normal skin; and **(f)** melanoma stained with monoclonal anti-non-functional P2X7 antibody. Panels **(g)** prostate PC3 cancer cell and **(h)** breast MCF7 cancer cell stained with the antibody visualised with cyanine 3 labelled secondary antibody. Field widths 0.5 mm (a-f) and 15 um (g,h).

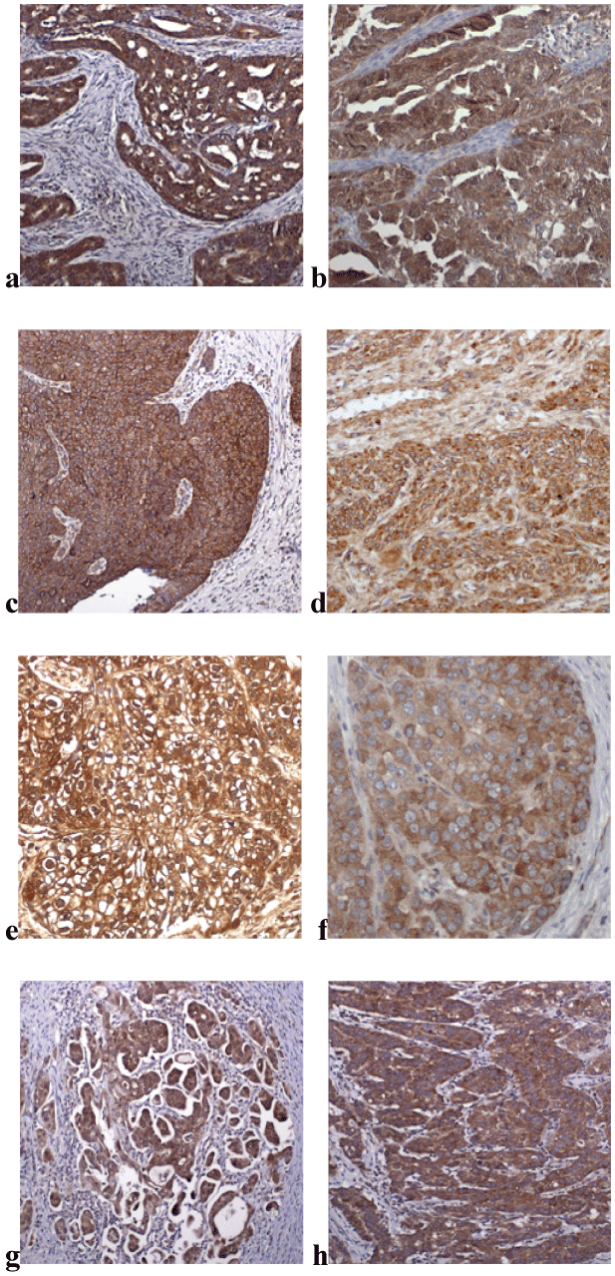

Figure 2: Sections of: **(a)** adenocarcinoma of the bowel; **(b)** serous ovarian cancer; **(c)** squamous cell cancer of the cervix; **(d)** endometrial cancer; **(e)** small cell lung cancer; **(f)** hepatocellular carcinoma; **(g)** transitional cell carcinoma of the bladder; and **(h)** Barrett's mucosa with adenocarcinoma stained with monoclonal anti-$nfP2X_7$ antibody. Field widths 0.5 mm.

Human cancers of non-epithelial cell origin were also found to express the $nfP2X_7$ receptors. Figure 3 shows examples of mesenchymal cancers gastrointestinal stromal tumour (Figure 3a) and endometrial stromal tumour (Figure 3b). Equally, other tumours derived from this cell origin such as Ewing's sarcoma express the receptors as do brain tumours such as oligodendrogliomas, glioblastomas and astrocytomas. An example of a rare pituitary

carcinoma is shown in Figure 3c. Mesothelioma, a cancer derived from pleural cells, is shown in Figure 3d and a trophoblastic tumour of placental origin is shown in Figure 3e. Solid tumours derived from blood cells such as mantle cell lymphomas also express the receptors. Examples shown include thyroid papillary (Figure 3f) and Hodgkin's lymphoma (Figure 3g).

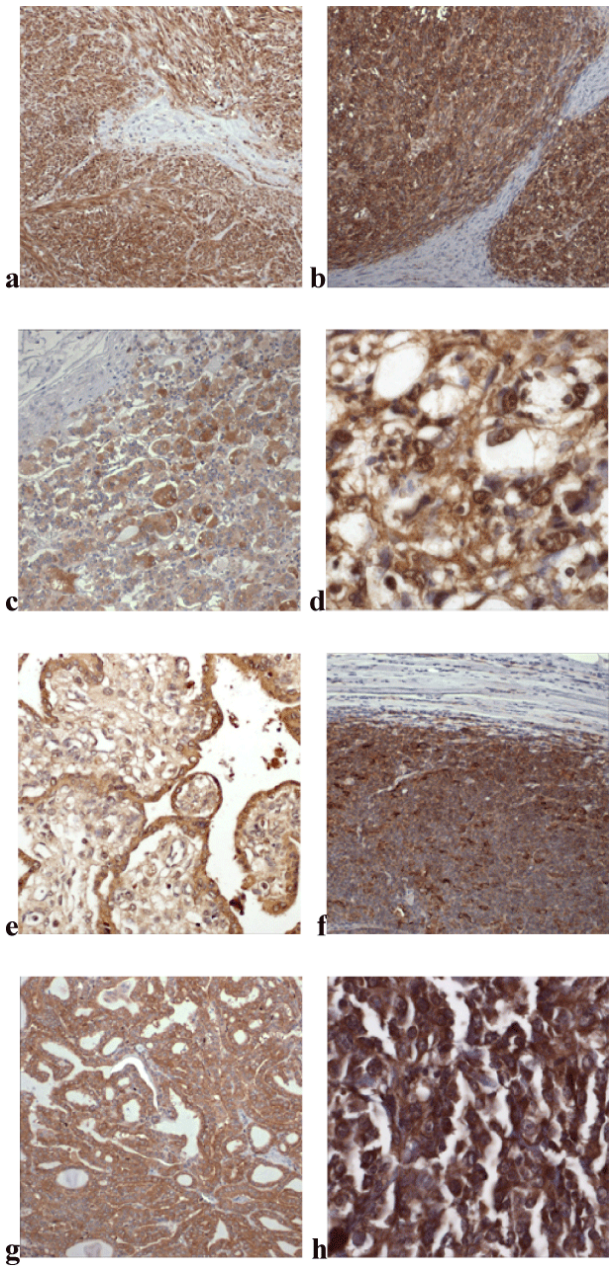

Figure 3: Sections of: **(a)** gastrointestinal stromal tumour; **(b)** endometrial stromal tumour; **(c)** pituitary cancer; **(d)** mesothelioma; **(e)** choriocarcinoma; **(f)** Hodgkin's lymphoma; **(g)** thyroid papillary; and **(h)** mouse B16/F10 melanoma stained with monoclonal anti-nfP2X$_7$ antibody. Field widths 0.5 mm (a-g) and 0.12 mm (h).

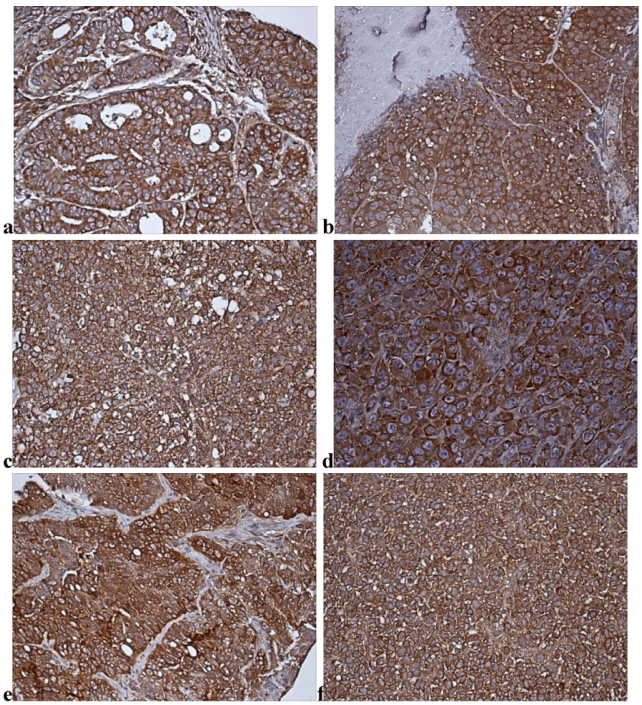

Figure 4: Sections of: **(a)** colon adenocarcinoma; **(b)** head and neck cancer; **(c)** testicular; **(d)** pancreatic; **(e)** gastric; **(f)** sarcoma showing consistent stain on the plasma membrane for nfP2X$_7$ receptors. Field widths 0.5 mm.

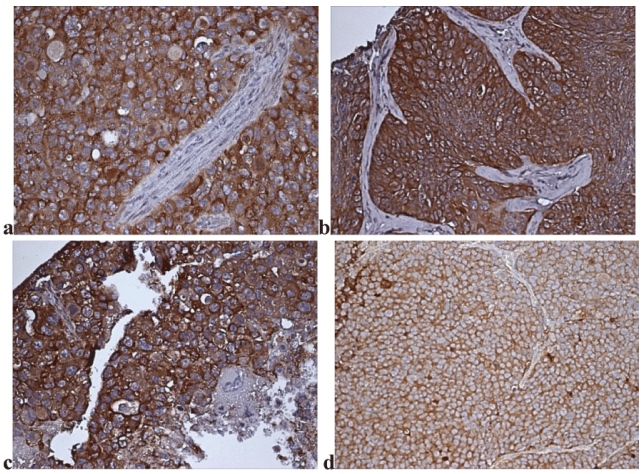

Figure 5: Sections of: **(a)** non small cell lung adenocarcinoma; **(b)** non small cell lung epidermoid; **(c)** large cell lung cancer; **(d)** small cell lung cancer **(d)** showing consistent stain on the plasma membrane for nfP2X$_7$ receptors. Field widths 0.5 mm.

The deployment of nfP2X$_7$ receptor on the plasma membrane in immortal cell lines indicates the strong potential for a causal role for nfP2X$_7$ in cancer whereby an apoptotic pathway activated by the cell fails because the receptor channel cannot function as a pore. Figure 4 shows examples of tumours from a tissue microarray stained under

identical conditions in which surface membrane receptor staining is evident on all types whether colon adenocarcinoma (Figure 4a), head and neck (Figure 4b), testicular (Figure 4c), pancreatic (Figure 4d), gastric (Figure 4e) or sarcoma (Figure 4f). The overall stain intensity and membranous pattern is clear regardless of cell size or type indicating similar receptor density on the surface of these and other cancer types. In Figure 5, four different subtypes of lung cancer are shown. Non small cell adenocarcinoma (Figure 5a), non small cell epidermoid (Figure 5b), large cell (Figure 5c) or small cell (Figure 5d) all show the average surface staining intensity is similar. The consistent strong membranous distribution indicates advanced cancers continue to deploy more $nfP2X_7$ potentially in a failed attempt to activate apoptosis.

Discussion

The appearance of a novel and ubiquitous $nfP2X_7$ receptor at the onset of human cancers of apparently all types indicates that the antibody probe can be used as an important adjunct to current diagnoses of cancer. There are indications that the receptor expression varies with tumour grade, at least in prostate cancer [11], providing the potential to differentiate between latent and aggressive forms of cancer. Certainly, very slow growing low grade prostate cancers exhibit a pattern of receptor expression that is almost entirely intracellular, while cases of invasive prostate cancer exhibit more plasma membrane and basal cell labelling together with a significantly elevated receptor density [29].

The monoclonal antibody to $nfP2X_7$ receptor is highly specific and does not bind on the surface of cells expressing only normal $P2X_7$ receptor. This specific pan-cancer diagnostic target may also have utility as a pan-cancer therapeutic target.

References

1. Bosch F, Rosich L (2008) The contributions of Paul Ehrlich to pharmacology: a tribute on the occasion of the centenary of his Nobel Prize. Pharmacology 82: 171-179.
2. Cancer Medicines in Development Presented by America's Biopharmaceutical Research Companies.
3. Nagy PV, Fehér T, Morga S, Matkó J (2000) Apoptosis of murine thymocytes induced by extracellular ATP is dose- and cytosolic pH-dependent. Immunol Lett 72: 23-30.
4. Coutinho-Silva R, Persechini PM, Bisaggio RD, Perfettini JL, Neto AC, et al. (1999) P2Z/P2X7 receptor-dependent apoptosis of dendritic cells. Am J Physiol 276: C1139-1147.
5. Chused TM, Apasov S, Sitkovsky M (1996) Murine T lymphocytes modulate activity of an ATP-activated P2Z-type purinoceptor during differentiation. J Immunol 157: 1371-1380.
6. Di Virgilio F, Chiozzi P, Ferrari D, Falzoni S, Sanz JM, et al. (2001) Nucleotide receptors: an emerging family of regulatory molecules in blood cells. Blood 97: 587-600.
7. Humphreys BD, Rice J, Kertesy SB, Dubyak GR (2000) Stress-activated protein kinase/JNK activation and apoptotic induction by the macrophage P2X7 nucleotide receptor. J Biol Chem 275: 26792-26798.
8. Grahames CB, Michel AD, Chessell IP, Humphrey PP (1999) Pharmacological characterization of ATP- and LPS-induced IL-1beta release in human monocytes. Br J Pharmacol 127: 1915-1921.
9. Slater M, Scolyer RA, Gidley-Baird A, Thompson JF, Barden JA (2003) Increased expression of apoptotic markers in melanoma. Melanoma Res 13: 137-145.
10. Slater M, Danieletto S, Pooley M, Cheng Teh L, Gidley-Baird A, et al. (2004) Differentiation between cancerous and normal hyperplastic lobules in breast lesions. Breast Cancer Res Treat 83: 1-10.
11. Slater M, Danieletto S, Gidley-Baird A, Teh LC, Barden JA (2004) Early prostate cancer detected using expression of non-functional cytolytic P2X7 receptors. Histopathology 44: 206-215.
12. Garcia-Marcos M, Perez-Andres E, Tandel S, Fontanils U, Kumps A, et al. (2006) Coupling of two pools of P2X7 receptors to distinct intracellular signaling pathways in rat submandibular gland. J Lipid Res 47: 705-714.
13. Rassendren F, Buell GN, Virginio C, Collo G, North RA, et al. (1997) The permeabilizing ATP receptor, P2X7. Cloning and expression of a human cDNA. J Biol Chem 272: 5482-5486.
14. Surprenant A, Rassendren F, Kawashima E, North RA, Buell G (1996) The cytolytic P2Z receptor for extracellular ATP identified as a P2X receptor (P2X7). Science 272: 735-738.
15. Virginio C, MacKenzie A, Rassendren FA, North RA, Surprenant A (1999) Pore dilation of neuronal P2X receptor channels. Nat Neurosci 2: 315-321.
16. Virginio C, MacKenzie A, North RA, Surprenant A (1999) Kinetics of cell lysis, dye uptake and permeability changes in cells expressing the rat P2X7 receptor. J Physiol 519 Pt 2: 335-346.
17. Morelli A, Chiozzi P, Chiesa A, Ferrari D, Sanz JM, et al. (2003) Extracellular ATP causes ROCK I-dependent bleb formation in P2X7-transfected HEK293 cells. Mol Biol Cell 14: 2655-2664.
18. Worthington RA, Smart ML, Gu BJ, Williams DA, Petrou S, et al. (2002) Point mutations confer loss of ATP-induced human P2X(7) receptor function. FEBS Lett 512: 43-46.
19. Barden JA, Sluyter R, Gu BJ, Wiley JS (2003) Specific detection of non-functional human P2X(7) receptors in HEK293 cells and B-lymphocytes. FEBS Lett 538: 159-162.
20. Jelassi B, Chantôme A, Alcaraz-Pérez F, Baroja-Mazo A, Cayuela ML, et al. (2011) P2X7 receptor activation enhances SK3 channels- and cysteine cathepsin-dependent cancer cells invasiveness. Oncogene 30: 2108-2122.
21. Qu Y, Dubyak GR (2009) P2X7 receptors regulate multiple types of membrane trafficking responses and non-classical secretion pathways. Purinergic Signal 5: 163-173.
22. Sluyter R, Wiley JS (2002) Extracellular adenosine 5'-triphosphate induces a loss of CD23 from human dendritic cells via activation of P2X7 receptors. Int Immunol 14: 1415-1421.
23. Gu BJ, Wiley JS (2006) Rapid ATP-induced release of matrix metalloproteinase 9 is mediated by the P2X7 receptor. Blood 107: 4946-4953.
24. Lopez-Castejon G, Theaker J, Pelegrin P, Clifton AD, Braddock M, et al. (2010) P2X(7) receptor-mediated release of cathepsins from macrophages is a cytokine-independent mechanism potentially involved in joint diseases. J Immunol 185: 2611-2619.
25. Sluyter R, Stokes L (2011) Significance of P2X7 receptor variants to human health and disease. Recent Pat DNA Gene Seq 5: 41-54.
26. Barden JA, Gidley-Baird A (2008) Antibodies to non-functional P2X7 receptor. US7326415.
27. Barden JA, Gidley-Baird A (2009) Novel P2X7 epitopes. WO2009033233.
28. Barden JA, Gidley-Baird A (2010) Anti-P2X7 peptides and epitopes. WO2010000041.
29. BPM09 (accession no. 06080101) European Collection of Cell Cultures (ECACC).

This article was originally published in a special issue, entitled: **"Tumor Immunology"**, Edited by Dr. David J Vigerust, Vanderbilt University School of Medicine, USA