Tumour antigen targeted monoclonal antibodies incorporating a novel multimerisation domain significantly enhance antibody dependent cellular cytotoxicity against colon cancer

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Abstract Tumour antigen targeted antibodies (mAbs) can induce natural killer (NK) cells to kill tumours through antibody dependent cellular cytotoxicity (ADCC) upon engagement of NK cell expressed FcγRIIIa. FcγRIIIa polymorphisms partially dictate the potency of the ADCC response. The high affinity FcγRIIIa-158-valine (V) polymorphism is associated with more potent ADCC response than the low affinity FcγRIIIa-158-phenylalanine (F) polymorphism. Because approximately 45% of patients are homozygous for the FcγRIIIa-158-F polymorphism (FF genotype), their ability to mount ADCC is impaired. We investigated whether...
1. Introduction

Cetuximab (Erbitux®), a monoclonal antibody (mAb) directed against the epidermal growth factor receptor (EGFR) and approved for the treatment of colorectal cancer, causes arrest of a KRAS dependent tumour proliferation pathway. While cetuximab improves progression-free survival in patients having tumours with wild type (WT) KRAS, it does not benefit the 30–60% of patients whose tumours bear codon 12 or 13 KRAS mutations. In 2009, the American Society of Clinical Oncology (ASCO) recommended that patients having KRAS-mutated tumours are not be offered the drug.

However, cetuximab may induce killing of even KRAS mutant tumours through ‘antibody dependent cellular cytotoxicity’ (ADCC) in some patients. High affinity engagement and aggregation of FcγRIIIa on natural killer (NK) cells by Immunoglobulin G, subclass 1 (IgG1) mAb Fc can cause NK cell activation, degranulation and tumour lysis. FcγRIIIa affinity is largely influenced by a single amino acid polymorphism at the 158 position, where substitution of valine (V) for phenylalanine (F) increases affinity and ADCC response.

For example, rituximab treated follicular lymphoma patients having the FcγRIIIa-158-VV genotype have a better prognosis than those with the FF genotype. Based on this indirect evidence, there is interest in developingFc γRIIIa-158-VV genotypes as targets and engaging multiple FcγRIIIa receptors could further enhance ADCC against colon cancer in vitro. Specifically, we generated a novel anti-epidermal growth factor receptor (EGFR) antibody (termed a stradobody™) consisting of an unmodified Fab sequence and two Immunoglobulin G, subclass 1 (IgG1) Fc domains separated by an isoleucine zipper domain and the 12 amino-acid IgG2 hinge. The stradobody™ framework induced multimerisation and was associated with increased binding to the EGFR and FcγRIIIa. From a functional perspective, when compared to an unmodified anti-EGFR mAb with a sequence identical to cetuximab (a commercially available anti-EGFR mAb), stradobodies™ significantly enhanced ADCC. These effects were observed using both KRAS wild type HT29 and KRAS mutant SW480 colon cancer cells as targets, and by NK cells obtained from healthy donors and a cohort of patients with colon cancer. These data suggest that high avidity cross-linking of multiple tumour surface antigens and multiple NK cell associated FcγRIIIa molecules can enhance ADCC and partially overcome impaired ADCC by FF genotype individuals in vitro.

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2. Materials and methods

2.1. Cell lines

HT29 and SW480 colon cancer cells (ATCC, Manassas, VA, United States of America (USA)) were cultured at 37°C with 5% CO2 in RPMI-1640 complete media (Mediatech Inc., Manassas, VA, USA) containing 10% heat-inactivated fetal bovine serum (FBS) (Atlanta Biologicals, Lawrenceville, GA, USA), 1% 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer (Mediatech Inc., Manassas, VA, USA), 1% L-glutamine (Life Technologies, Grand Island, NY, USA) and 1% penicillin–streptomycin (Life Technologies).

2.2. Antibodies for ADCC

Human IgG1 isotype control antibody was obtained from Sigma (Sigma, St. Louis, MO, USA). Cetuximab (Erbitux®) was obtained from the University of Maryland Cancer Center Pharmacy (Baltimore, MD, USA). The anti-EGFR mAbs and stradobodies™ were generated by Gliknik Inc. (Gliknik Inc., Baltimore, MD, USA).

2.3. Biacore

Direct immobilisation of the commercially available FcγRIIIa (R&D Systems, Minneapolis, MN, USA) was not possible because the regeneration buffer required to disrupt binding damaged the chip surface. Therefore, an indirect capture method was used according to manufacturer’s instructions for a Biacore 3000 instrument. After immobilising 12000RU of anti-histidine onto a CM5 chip, 120RU of histidine-tagged FcγRIIIa-158-V (RnD Systems) was captured with a 1-min injection at 10 μl/min on one flow cell. Samples serially diluted from 1000 nM to 1.6 nM with HBS-EP were injected at 50 μl/min for 3 min. Following a 180-s dissociation phase, the chip was regenerated with 10 mM glycine, pH 1.5.
We also measured binding to the FcγRIIIa-158-V and F polymorphic receptor variants (Gliknik Inc., Baltimore, MD, USA), using a kinetic titration series method. After immobilisation, samples serially diluted from 625 nM to 1 nM, were injected in ascending order. Each injection was followed with a 180-s dissociation phase. After each titration, the chip was fully regenerated.

A second flow cell was used as reference. Blank control cycles were performed for each series. Unless otherwise specified, all reagents and equipment were from GE Healthcare; Piscataway, NJ.

2.4. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE)

We used 4–12% Nupage Novex Bis–Tris mini-gels for the non-reduced proteins and 12% gels (Invitrogen, Grand Island, NY, USA) for the reduced proteins as previously described.

2.5. Flow cytometry-based binding assay

Cells were stained with PE-conjugated rat anti-EGFR mAb, clone ICR10 (Abcam, Cambridge, MA, USA) or rat IgG2A isotype antibody (BD Biosciences, San Jose, CA, USA) according to manufacturer’s instructions. HT29 cells were incubated with escalating concentrations (0.1 μg/mL, 1 μg/mL, 10 μg/mL and 100 μg/mL) of unlabeled mAbs at 4 °C for 30 min, followed by incubation with 0.1 μg/mL APC-conjugated Erbitux®. Analysis was performed on a BD LSR II flow cytometer (BD Biosciences) and mean fluorescence intensity (MFI) values in the gated region of analysis were calculated using BD FACSDiva™ (BD Biosciences) and FlowJo (Tree Star, Ashland, OR, USA) software.

2.6. Purification of NK cells

Healthy human buffy coats were purchased from Biological Specialty Corporation (Colmar, PA, USA). Peripheral blood mononuclear cells (PBMCs) were isolated using Ficoll-Paque PLUS density gradient centrifugation (GE Healthcare, Uppsala, Sweden). NK cells were isolated using a MACS® human NK cell negative selection isolation kit (Miltenyi Biotec, Auburn, CA, USA) according to manufacturer’s instructions.

2.7. FcγRIIIa genotyping

Genotyping for FcγRIIIa single-nucleotide polymorphisms (SNP) rs396991 was done as previously described. For FcγRIIIa, the gene-specific forward primer 5’-AGTT-CATCATAATCTGACTCCT-3’ and reverse primer 5’-ACCTTGAGTGATGTTGTTCA-3’ were used.

Products were run on a ABI Prism 3130 DNA sequencer and data were analysed using the GeneMapper Analysis software (Applied Biosystems, Grand Island, NY, USA).

2.8. 51Cr-release assay

The assay was performed as described by Taylor et al. NK cells were added to targets at E:T ratios ranging from 50:1 to 12.5:1. Test antibodies were at a concentration of 10 μg/mL. Results were conveyed as the percentage of specific lysis using the following formula: % cytotoxicity = ([Experimental CPM – Spontaneous CPM]/[Maximum CPM – Spontaneous CPM]) × 100.

The Grubbs’ test was used to exclude individual outlier data points more than 1.96 sample standard deviations from the sample mean in any of our triplicate data.

\[ Z = \frac{\text{mean-value}}{\text{SD}} \]

2.9. Complement dependent cytotoxicity (CDC) assay

Target cells (1 × 10^5) were incubated in 96-well plates with test antibodies (1–1000 ng/ml) for 1 h at 37 °C in the presence of rabbit complement (Pel-Freez Biologics) at final dilutions of 1:10 for HT29 and 1:20 for SW480. Cytotoxicity was measured by FACS analysis of annexin V/7-AAD fluorescence (Annexin V-PE apoptosis detection kit I, BD Biosciences) according to the manufacturer’s instructions.

2.10. Patient studies

Colon cancer patients were recruited at the Baltimore VA Medical Center and the University of Maryland Medical Center under an Institutional Review Board (IRB) protocol approved protocol. NK cells were isolated from 150 mL of blood. Some donors did not yield enough effector cells to test with all antibodies.

2.11. Antibody design

DNA fragments encoding Erbitux® heavy and light chains were synthesised at BlueHeron (Bothell, WA, USA) based on cetuximab amino-acid sequences obtained from the Drugbank database (http://www.drugbank.ca/drugs/DB00002). The sequence differs by one amino acid residue from a sequence in the IMGT data base (http://www.imgt.org). Heavy (HC) and light chain (LC) DNA fragments were cloned into pOptivec (HC) and pcDNA3.3 (LC) (Invitrogen, Carlsbad, CA, USA) by TA cloning and sequence confirmed. The stradobody™ with the structure of two IgG1 Fc domains separated with an isoleucine zipper domain and the 12 amino-acid IgG2 hinge was made by a combination of overlapping polymerase chain reaction (PCR) and restriction digestion and sequence confirmed.
Proteins were generated by transient transfection in HEK293 cells, followed by purification on a AKTexpress system using a HiTrap MabSelect protein A affinity column and gel filtration on a HiPrep column (GE Healthcare Lifesciences, Piscataway, NJ).

2.12. Statistical analysis

Wilcoxon signed rank tests were used to compare cytotoxicity between the different antibodies. \( p < 0.05 \) was considered statistically significant. No adjustments for multiple comparisons were made. For CDC data, differences in cytotoxicity between the anti-EGFR mAb and stradobody were evaluated using a two-tailed Student’s \( t \)-test.

3. Results

3.1. Anti-EGFR stradobodies exist as homodimers and single order multimers

To develop antibody-like molecules containing multiple Fc and Fab fragments, anti-EGFR stradobodies were engineered to incorporate an unmodified Fab sequence and two IgG1 Fc domains separated by an iso-leucine zipper (ILZ) and the IgG2 hinge (2H) multimerisation domains (MDs) (Fig. 1a).\(^{25} \) The MDs were chosen based on screening of numerous compounds. Our published studies have demonstrated that these protein sequences can effectively multimerise fully recombinant murine IgG2a Fc.\(^{22} \) In order to eliminate concerns that production differences between the commercial mAb, cetuximab (Erbitux\(^{10} \)) and the stradobody might confound our results, we generated recombinant cetuximab (anti-EGFR mAb) as a control. SDS–PAGE confirmed that IgG1 isotype control and unmodified anti-EGFR mAb exist primarily as homodimers with a MW of 150 KDa. In contrast, the stradobody exists as two independent bands: (1) The homodimer which contains two Fc domains, and (2) a presumed dimer of dimers, bearing four Fc components and two Fab fragments (Fig. 1b). Importantly, because the non-reduced anti-EGFR mAb produced by transient expression had a different banding pattern than IgG1 isotype control, we analysed migration on a reducing gel, which confirmed that the banding configurations were equivalent (Fig. 1b).

3.2. Stradobodies efficiently block Erbitux binding to the EGFR

In order to confirm that the anti-EGFR mAbs and stradobodies were specifically binding the EGFR at the same epitope as cetuximab (Erbitux\(^{10} \)), we performed a flow cytometry based competition assay. Preincubation of EGFR positive HT29 cells with either the anti-EGFR mAbs or stradobodies™ blocked Erbitux\(^{10} \) binding, however, the stradobodies™ blocked at much lower concentrations (Fig. 1c). Furthermore, at the highest concentrations evaluated, the recombinant mAb did not completely block Erbitux\(^{10} \) binding. In contrast, the stradobodies™ demonstrated 100% inhibition at the highest concentration, and near 100% inhibition at 2-log order lower dose. These data confirm that the anti-EGFR mAbs and stradobodies™ share an overlapping binding site with Erbitux\(^{10} \), but the stradobodies™ bind more efficiently than non-multimerising mAbs.

3.3. Stradobody™ Fc domain multimerisation increases affinity for Fc\(\gamma\)RIIIa

Using a Biacore assay, we confirmed that the stradobody™ framework increases Fc affinity for Fc\(\gamma\)RIIIa-158-F and Fc\(\gamma\)RIIIa158-V polymorphic receptors. The commercially available form of Fc\(\gamma\)RIIIa carries the high affinity V polymorphism, and there is no commercially available F phenotype receptor. Biacore assay confirmed that the anti-EGFR mAb and stradobody bound Fc\(\gamma\)RIIIa with greater affinity than IgG1 (KDa: IgG1 = 2.80 \(\times\) \(10^{-6}\) M, anti-EGFR mAb: 1.39 \(\times\) \(10^{-8}\) M, anti-EGFR stradobody™ 5.9 \(\times\) \(10^{-9}\) M, Fig. 2). To determine if the differences in binding affinity between the anti-EGFR mAb and stradobody™ are more pronounced against Fc\(\gamma\)RIIIa-158-F phenotype receptors, we generated a recombinant Fc\(\gamma\)RIIIa-158-F receptor, as well as a Fc\(\gamma\)RIIIa158-V receptor for comparison to the commercial product. These recombinant receptors were less stable than the optimised, commercially available Fc\(\gamma\)RIIIa158-V receptor and degraded after repeated injection and regeneration cycles. Therefore, it was necessary to use a single cycle kinetic model for the Biacore assay.\(^{21} \) Nevertheless, binding to our recombinant Fc\(\gamma\)RIIIa158-V receptor was similar to that observed using the commercially available Fc\(\gamma\)RIIIa-158-V receptor (KDa: IgG1 = 7.13 \(\times\) \(10^{-7}\) M, anti-EGFR mAb: 9.84 \(\times\) \(10^{-9}\) M, anti-EGFR stradobody™ 1.58 \(\times\) \(10^{-9}\) M, Supplemental Fig. 1). Importantly, the stradobody™ bound the Fc\(\gamma\)RIIIa158-F phenotype receptor with more than one log order higher affinity than the anti-EGFR mAb (KDa: IgG1 = 3.08 \(\times\) \(10^{-6}\) M, anti-EGFR mAb: 3.15 \(\times\) \(10^{-8}\) M, anti-EGFR stradobody™ 7.95 \(\times\) \(10^{-9}\) M, Supplemental Fig. 1). These data suggest that the stradobody™ might have significant potential to amplify ADCC.

3.4. Stradobodies increase ADCC

Since the stradobodies™ engage multiple Fc\(\gamma\)RIIIa receptors and multiple EGFR targets, we hypothesised that they would enhance ADCC against colon cancer cells in vitro. The KRAS WT HT29 cells were generally more susceptible to ADCC than KRAS mutant SW480 cells.
Against both cell lines, however, the stradobody™ was significantly more effective at increasing ADCC compared to the unmodified anti-EGFR mAb, even when using FcγRIIIa-158-FF genotype NK cells (p < .01, Fig. 3). Furthermore, stradobodies™ were able to induce CDC against both tumour cell lines, suggesting this mechanism may also be relevant in the clinical setting (Supplemental Fig. 2).

3.5. Stradobodies™ enhance ADCC by colon cancer patients

Unlike healthy humans, cancer patients have impaired NK cell function and ADCC response.26–28 As a preliminary step towards clinical translation, we determined if stradobodies™ could enhance ADCC by NK cells from colon cancer patients. Eleven of fourteen cancer patient donors tested had stage III or IV disease (Table 1). Even so, the stradobody™ framework significantly enhanced ADCC compared to the unmodified anti-EGFR mAb (p = .0.008, Fig. 4). Although there were not enough patients to perform statistical analysis based on FcγRIIIa genotype (FF versus VF/VV), the data appear similar to those generated using healthy donor NK cells, providing preliminary evidence that stradobodies™ may have therapeutic potential.

4. Discussion

The FcγRIIIa expressed by FF genotype individuals has lower affinity for IgG1 Fc and limited potential to induce ADCC. In this report, we demonstrate that anti-EGFR targeted, IgG1-based stradobodies™ presenting multivalent Fab binding sites and polyvalent Fc significantly enhance ADCC against colon cancer by FF genotype NK cells. Importantly, these findings are not restricted to NK cells from healthy donors, but appear to be conserved in NK cells from colon cancer patients.

We first demonstrated that the stradobody™ forms multimers. Variable domain (Fab) multimerisation allowed the stradobody™ to block Erbitux® binding to...
Fig. 2. The stradobody framework significantly increases affinity for FcγRIIIa-valine (V) polymorphic receptors. The IgG1 isotype control, anti-epidermal growth factor receptor (EGFR) monoclonal antibody (mAb) and stradobody™, were injected over FcγRIIIa-V for 180 s in ascending order (1.6, 8, 40, 200 and 1000 nM). All samples were injected in duplicate. Increased affinity is indicated by higher $k_a\cdot$, lower $k_d\cdot$ and lower $K_D\cdot$. Rate constants were measured using the bivalent binding model from the BIAevaluation software, v3.1.

![Graph showing k values](image)

**HT29 TARGETS**

- **FF**
  - IgG1
  - Anti-EGFR
  - Stradobody™
  - *p=0.002

- **VF/VV**
  - IgG1
  - Anti-EGFR
  - Stradobody™
  - *p=0.001

**SW480 TARGETS**

- **FF**
  - IgG1
  - Anti-EGFR
  - Stradobody™
  - N=11

- **VF/VV**
  - IgG1
  - Anti-EGFR
  - Stradobody™
  - N=16

Fig. 3. The stradobody™ enhances antibody dependent cellular cytotoxicity (ADCC). ADCC against KRAS-WT HT29 and KRAS mutant SW480 cells by FcγRIIIa-158-FF and FcγRIIIa158-VF/VV donor natural killer (NK) cells is shown in culture with IgG1 isotype control, anti-epidermal growth factor receptor (EGFR) monoclonal antibody (mAb) and the stradobody™. The horizontal line (—) indicates the median. The lower box represents the 25–50% quartile, while the upper box represents the 50–75% quartile. The absolute numbers of donors tested with each antibody are shown, as some donors did not yield enough NK effectors to test with all mAbs. * indicates comparison against anti-EGFR mAb.
EGFR expressing cells more efficiently than unmodified anti-EGFR mAbs. Multimerisation also significantly increased Fc affinity for FcγRIIIa, including those variants with the low affinity F polymorphism. The ability to multimerise makes the stradobody™ distinct from previously described Fc modified, tumour antigen targeted mAbs. The design of ADCC-inducing mAbs has largely focused on altering the mAb Fc fragment through protein and/or glyco manipulation to increase affinity for individual FcγRIIIa receptors. However, recent data suggest that cytotoxicity cannot be increased beyond a certain level through modifications that increase affinity of a single Fc domain for a single FcγRIIIa. For example, Repp and colleagues showed that doubly engineered mAbs incorporating both amino acid substitutions [S239D/A330L/I332E] and Fc defucosylation bind FcγRIIIa better than mAbs containing either modification alone, but ADCC is not further enhanced.

It is conceivable that by combining the stradobody™ MD with established Fc domain modifications, higher affinity crosslinking of multiple NK cell FcγRIIIa receptors could further enhance ADCC. In addition, established Fc domain modifications do not cause Fab multimerisation, which allows higher affinity engagement of tumour antigens as a secondary mechanism to increase ADCC.

In addition to their improved affinity, stradobodies™ were more effective than unmodified anti-EGFR mAbs in mediating ADCC by healthy donor NK cells against both KRAS WT and mutant colorectal cancer cell lines. Furthermore, since cancer patients often have impaired

Table 1: Genotype and tumour-node-metastasis (TNM) staging of colon cancer donors.

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a Patient presented with liver metastasis several months after resection of primary at outside institution.
b Patient had rectal cancer and underwent staging endorectal ultrasound. Clinical stage was T3N1, so the patient received neoadjuvant chemoradiation before surgery. Final surgical pathologic stage was T2N0 after downsizing from chemoradiation, but management is based on preoperative clinical staging. Blood was drawn several weeks after completion of neoadjuvant chemoradiation (non-immunosuppressed state) prior to resection.
c Patient has biopsy proven recurrent rectal cancer. Positron emission tomography (PET)–computerised tomography (CT) shows enhancement in sacrum and acromion. X-rays show lytic lesions in acromion, consistent with bony metastasis. Clinically being managed as stage IVa metastatic disease.
d Patient has recurrent rectal cancer with liver metastases.
e Patient initially underwent transanal excision based on preoperative ultrasound staging indicating early stage (T1) tumour. Final surgical pathology revealed T2 lesion with lymphovascular invasion, upstaging to stage II. The patient underwent completion abdominoperineal resection, confirming 3/22 nodes were positive. This resulted in upstaging to Stage IIIa.

Fig. 4. Stradobodies™ enhance antibody dependent cellular cytotoxicity (ADCC) by natural killer (NK) cells from cancer patients. ADCC by NK cells from colon cancer patients against SW480 targets is shown in culture with IgG1 isotype control, anti-epidermal growth factor receptor (EGFR) monoclonal antibody (mAb), and the stradobody™. Some donors did not yield enough NK cell effectors to test with all antibodies. (− = median lysis) * indicates comparison against anti-EGFR mAb.

EGFR expressing cells more efficiently than unmodified anti-EGFR mAbs. Multimerisation also significantly increased Fc affinity for FcγRIIIa, including those variants with the low affinity F polymorphism. The ability to multimerise makes the stradobody™ distinct from previously described Fc modified, tumour antigen targeted mAbs. The design of ADCC-inducing mAbs has largely focused on altering the mAb Fc fragment through protein and/or glyco manipulation to increase affinity for individual FcγRIIIa receptors. However, recent data suggest that cytotoxicity cannot be increased beyond a certain level through modifications that increase affinity of a single Fc domain for a single FcγRIIIa. For example, Repp and colleagues showed that doubly engineered mAbs incorporating both amino acid substitutions [S239D/A330L/I332E] and Fc defucosylation bind FcγRIIIa better than mAbs containing either modification alone, but ADCC is not further enhanced. It is conceivable that by combining the stradobody™ MD with established Fc domain modifications, higher affinity crosslinking of multiple NK cell FcγRIIIa receptors could further enhance ADCC. In addition, established Fc domain modifications do not cause Fab multimerisation, which allows higher affinity engagement of tumour antigens as a secondary mechanism to increase ADCC.

In addition to their improved affinity, stradobodies™ were more effective than unmodified anti-EGFR mAbs in mediating ADCC by healthy donor NK cells against both KRAS WT and mutant colorectal cancer cell lines. Furthermore, since cancer patients often have impaired
NK cell function,26–28 we also evaluated if stradobodies™ could enhance ADCC by NK cells from colorectal cancer patients. The results were very similar to those obtained using healthy donor NK cells. Although the number of cancer patients was low, these results are encouraging, especially since most of the patients had stage III (node positive) or IVa (metastasis to a single organ) disease. These data suggest that stradobodies™ may have translational therapeutic potential in cancer patients.

Since stradobodies™ are larger than traditional mAbs, they may traffic differently or be cleared more rapidly from circulation.29 Our data showing that stradobodies™ induce CDC in vitro, raise the possibility that CDC may be another mechanism for inducing tumour regression. In vivo data ultimately will be required to confirm the potential for clinical efficacy. Execution of in vivo studies is complicated by the lack of robust, commercially-available, animal models that target human tumour cell lines and employ NK cells expressing the human FcγRIIIa polymorphisms. Even so, our in vitro data suggest that stradobody™ MD may be novel and relevant advance in the design of the ADCC inducing cancer therapeutics.

Role of the funding source

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Conflict of interest statement

Dr. Strome is a Cofounder and Major stockholder in Gliknik Inc., a biotechnology company. He also receives royalties for intellectual property, related to 4-1BB and B7-H1, licensed by the Mayo Clinic College of Medicine to third parties. Gliknik Inc. is both a co-inventor and the exclusive licensee from the University of Maryland, Baltimore, of the stradobody™ technology platform. Dr. Block, Dr. Olsen, Mr. Mérigeon, and Ms. Chan are employees of Gliknik Inc. All other authors have no competing interests.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ejca.2013.06.009.

References


