Cytolytic tests with hyperimmune patient sera is a good prognostic tool in racotumomab immunotherapy in advanced non-small cell lung cancer

Necdet Uskent¹, Nil Molinas Mandel², Zafer Gulbas³, Gulcan Baloglu⁴, Barkin Berk⁵, Ruchan Uslu⁶, Aziz Yazar⁷, Huseyin Baloglu⁸

¹ Department of Medical Oncology, Anadolu Health Center, Kocaeli, Turkey
² Department of Medical Oncology, School of Medicine, Koç University, Istanbul, Turkey
³ Department of Hematology and Bone Marrow Transplantation and Cellular Therapy Unit, Anadolu Health Science Center, Kocaeli, Turkey
⁴ Department of Biochemistry, Anadolu Health Science Center, Kocaeli, Turkey
⁵ School of Medicine, Medipol University, Istanbul, Turkey
⁶ Department of Medical Oncology, School of Medicine, University of Ege, Izmir, Turkey
⁷ Department of Medical Oncology, Acıbadem University Hospital, Kozyatagı, Istanbul, Turkey
⁸ Department of Pathology, Anadolu Health Science Center, Kocaeli, Turkey

Abstract: Aberrant accumulation of a specific sialic acid has been shown to exist in many human malignant cell membranes termed as N-glycolyl neuraminic acid (NeuGc). This particular ganglioside do not normally exist in normal human cells, due to the lack of an enzyme (cytidine monophospho-N-acetyl-neuraminic acid) which is responsible for the synthesis of N-glycolyl neuraminic acid. The aberrant expression of NeuGcGM3 ganglioside in the cell surface of certain human tumors, made this molecule an attractive target for immunotherapy. By using 14F7 monoclonal antibody directed to identify NeuGcGM3 in the tumor tissue, it is possible to select patients for anti-NeuGcGM3 immunotherapy. Racotumomab is an anti-idiotypic vaccine, being a mirror image of NeuGcGM3 mimics this ganglioside and triggers an immune response. Antibodies reactive to NeuGcGM3 ganglioside in the vaccinated patient’s sera have cytotoxic anti-tumor properties which can be assessed in L1210 cell line, expressing this ganglioside.

In this study, we monitored 12 patients with advanced non-small cell lung cancer (NSCLC) who are on racotumomab vaccine maintenance following chemotherapy. Cytotoxic tests with vaccinated patients’ sera were performed using L1210 cell lines at the 3rd, 6th, 9th, and 12th months of vaccination and the results were compared with clinical outcomes. Serum antibodies to NeuGcGM3 ganglioside were also checked before initiation and thereafter with the same intervals. The aim of the study was to investigate the value of antibodies and cytotoxic test as biomarkers for treatment outcome. Our observation confirmed that consistently higher cytotoxicity rates in the cell culture correlated with better progression free survivals of the patients who are on racotumomab maintenance.

Keywords: racotumomab; cytotoxic tests with hyperimmune patients’ sera; non-small cell lung cancer; cancer vaccines


*Correspondence to: Necdet Uskent, Department of Medical Oncology, Anadolu Health Center, Kocaeli, Turkey; necdet.uskent@anadolusaglik.org

Received: 8th April 2017; Accepted: 7th August 2017; Published Online: 23rd October 2017

Introduction

There is no expression of NeuGcGM3 ganglioside in normal human tissues, apart from minute amounts in brain tissue, due to the incapability of human cells synthesizing the N-glycoly neuraminic acid (NeuGc). It is due to the lack of an enzyme which is responsible for the synthesis of this particular sialic acid in humans[1]. The aberrant expression of NeuGcGM3 ganglioside in the cell surface of certain human tumors, made this molecule an attractive target for immunotherapy. By using 14F7 monoclonal
Cytolytic tests with hyperimmune patient sera is a good prognostic tool in racotumomab immunotherapy in advanced non-small cell lung cancer

Antibodies directed to identify NeuGcGM3 in the tumor tissue, it is possible to select patients for anti-NeuGcGM3 immunotherapy[2]. Racotumomab is an anti-idiotypic vaccine, being a mirror image of NeuGcGm3 mimics this ganglioside and triggers an immune response. Antibodies reactive to NeuGcGM3 ganglioside in the vaccinated patient’s sera have cytotoxic anti-tumor properties which can be assessed in L1210 cell line, expressing this ganglioside[1]. Aberrant accumulation and over-expression of NeuGcGM3 has been detected in human malignant tumors such as lung cancers, breast cancers, gliomas, urinary bladder tumors and hepatocellular cancers[4]. This aberrant expression of NeuGcGm3 is commonly detected in the surface of malignant cells, although cytoplasmic involvement was also reported. In a study, intense expression of this ganglioside in the periphery of nucleus was detected. Authors explained this finding as the possible synthesis of NeuGcGM3 in the golgi apparatus and intracellular movement of this molecule within the cell[5,6].

There are numerous hypothesis explaining the existence of NeuGcGM3 in human malignancies, from changing dietary habits to the modified metabolism of malignant cells as a result of genetic mutations, and due to the hypoxic conditions of tumor[7]. Yin and co-workers showed in their study that hypoxic conditions resulted in over expression of sialic acid transporter sialin, and induced cancer-associated gangliosides in human cancer cells[8].

It was shown that, incorporation of NeuGcGM3 into the cell membrane of T-lymphocytes down-regulates the CD4 expression and interfere in the functions of both CD4 and CD25 T-lymphocytes along with dendritic cells[9]. Conclusively, these factors could lead to tumor-induced suppression by inhibiting the specific immune response.

NeuGcGM3 is capable to bind to the extracellular domain of the epidermal growth factor receptor (EGFR). This binding may provoke EGFR system activation mediated by the epidermal growth factor ligand (EGF). Activation of EGF in the presence of over expressed NeuGcGM3 constitutes a more aggressive immunophenotype in non-small cell lung cancer (NSCLC)[10]. Combination of anti-EGF plus anti-NeuGcGM3 immunotherapies are currently under investigation[11].

Abnormal accumulation of NeuGcGm3 in selected human malignant tumor cells, made this molecule an attractive target for cancer immunotherapy, minimizing the risk of potential damage to healthy tissues, since normal human cells do not carry this ganglioside[12]. The immunostaining with the 14F7 IgG1 monoclonal antibody is a highly specific tool for the detection of NeuGcGM3 ganglioside in the tumor tissue.

The immunohistochemical detection of NeuGcGM3, allows the potential selection of patients for specific therapy with anti-idiotypic racotumomab cancer vaccine[3]. Racotumomab is an anti-idiotypic monoclonal antibody raised against NeuGcGM3 in combination with aluminum hydroxide, as an adjuvant. Alum enhances the production of antibodies, stimulates monocytes to produce proinflammatory cytokines, and slower the release of antigen from the injection site[13,14].

The production process of racotumomab anti-idiotypic mouse monoclonal antibody, involves the immunization of Balb/c mice with NeuGcGM3 ganglioside, followed by isolation of IGM monoclonal antibody (Ab1). This Ab1 antibody was again used in mice to generate an IgG1 monoclonal antibody (Ab2) later named as racotumomab which has a high affinity towards Ab1 idiotype (Figure 1). Antibodies reactive to NeuGcGM3 ganglioside in the vaccinated patient’s sera have cytotoxic anti-tumor properties which can be monitored in L1210 cell line expressing this ganglioside.

In human healthy donors, it was found that naturally occurring antibodies against NeuGcGM3 are able to recognize and kill tumor cells expressing this antigen[15]. Antibodies reactive to NeuGcGM3 ganglioside in the vaccinated patient’s sera have cytotoxic anti-tumor properties which can be enumerated in L1210 cell line expressing this ganglioside[3]. In this study, we monitored 12 patients with advanced non-small cell lung cancer who have been treated with racotumomab vaccine, performed cytotoxic tests with hyperimmune patient’s sera in L1210 cell lines, at the 3rd, 6th, 9th, and 12th months of vaccination and matched the results with clinical outcomes.

Materials and methods

Study design and selection of patients

The primary end point of the study was to investigate the value of serum anti-NeuGcGM3 antibodies and cytotoxic tests (cytotoxic properties of vaccinated patients’ sera in cell culture) as biomarkers of treatment efficacy or failure. We also investigated the value of these two markers in connection with progression free survival. The secondary
end point of the study was to investigate the relationship between the intensity of NeuGcGM3 ganglioside expression in the tumor tissue and cytotoxicity results. Out of 30 stage III-IV non-small cell lung cancer patients who were eligible for racotumomab maintenance treatment, 12 patients whose tumor tissue expressed NeuGcGM3 with 14F7 monoclonal antibody included in the study (Figure 2).

A total of 10 stage IV patients were at least in partial remission after first line chemotherapies consisting of platinum doublets. None of the patients had sensible mutations like EGFR, ROS-1 and EML-ALK translocation. These patients received racotumomab as switch maintenance. Only 2 patients in stage IIIA/B received racotumomab as adjuvant, postoperatively (stage IIIA) and following chemoradiotherapy (stage IIIB). The route of administration was intradermal, each dose divided into four different anatomic sites in the medial surfaces of arms, thighs or abdomen. The vaccination program consisted of two phases. In the induction phase, 5 doses were administered 2 weeks apart, followed by the maintenance phase, where 10 more doses were administered monthly. Patients without progression were encouraged to continue the vaccine beyond the 15th application.

The patients have been treated in 4 different oncology
Cytolytic tests with hyperimmune patient sera is a good prognostic tool in racotumomab immunotherapy in advanced non-small cell lung cancer

centers (ANADOLU Health Center, KOC University, EGE University and ACIBADEM University). However, all the lab work, including antibody detection, tissue NeuGcGm3 expression and cytotoxic tests with patients’ sera were performed at ANADOLU Health Center. Cytotoxic tests from the same sera’s were repeated at MEDIPOL University, for confirmation. In case of a conflicting result, tests were run for the 3rd time, in each institution, until reconciliation has been reached. The immunostaining was performed with the 14F7 monoclonal antibody in all patients’ tumor tissue.

**Laboratory Analysis**

**Immunohistochemistry**

For the detection of expression of NeuGcGM3 in the tumor paraffin blocks, the murine monoclonal antibody 14 F7 was used. This antibody was obtained from mice that were immunized with purified gangliosides and supplied by the manufacturer institute (CIM-Center of Molecular Immunology, Havana, Cuba). Detection of the ganglioside in the tumor tissue was done by immunohistochemistry (IHC) with 20 µg/mL, biotinylated anti-IgG immune serum and a streptavidin-peroxidase commercial kit that uses diaminobenzidine as a peroxidase substrate. Immunoreactivity on the sample was evidenced as a brown staining. 5µm-thick sections of tissue was used to observe the expression of NeuGcGM3[6].

**Cytotoxicity test**

Antibodies to NeuGcGM3 have been detected and cytotoxic tests was done with hyperimmune patient’s sera in L1210 cell lines expressing N-Glycol GM3, using BD FACS Calibur Flow Cytometry System, BD influx flow machine and BD FACS software, at the time of initiation (day zero), and at the 3rd, 6th, 9th and 12th months of vaccination. Both pre-immune and hyperimmune patients’ sera were incubated with the L1210 murine lymphocytic leukemia cell line. The L1210 murine lymphocytic leukemia cell line expressing high levels of NeuGcGM3 was provided from ATCC (American Type Culture Collection Co.). Cells were grown in DMEM medium supplemented with 10% of heat-inactivated fetal bovine serum (Gibco), 2 mmol/L L-glutamine and maintained at 37°C for 3 h. To determine the cell death (cell-kill) percentage, propidium iodine (PI; Sigma, Aldrich) was used at a final concentration of 10/ mL cytotoxicity was evaluated by flow cytometry using the PI exclusion assay. This assay was also repeated with normal controls sera. Cytotoxic activity over cut off value of 30% increased cell kill compared to basal level (day 0 preimmune sera), when available, or against negative control (healthy donor’s sera) considered positive. Cells without immune sera (cell alone) were also used as a double negative control. Results of cytotoxic tests as a prognostic tool and clinical outcome of the patients were compared. The assessment of the potential predictive value of NeuGcGM3 expressions in the tumor tissue for efficacy outcomes was also investigated.

**Scanning electron microscopy**

Scanning electron microscopy (Zeiss Sigma 500) photographs of antibody induced apoptosis in L1210 cell culture were performed at 9 EYUL University Institutes of Biomedicine and Genom. For the preparation of cells to electron microscopic photographing, L1210 cells in 100 mL RPMI 1640 culture medium supplemented with 1% fetal calf serum (FCS) were incubated with patients’ sera for 2 h at 37°C, washed three times with PBS, and then fixed with 3.2% glutaraldehyde in 0.1 mol/L sodium phosphate buffer (pH 7.4) at 4°C for 1 h. These cells were postfixed for 1h in 1% OsO4, washed three times with phosphate buffer saline (PBS), and dehydrated in ethyl alcohol. Cells were mounted onto a metallic stub and coated (Quorum Q150R) with 5 nm thick gold before analyzed by Zeiss Sigma 500.

**Ethics statement**

The Institutional Review Board of Ethical Committee of MEDIPOL University granted approval for this study (Approval No: 10840098-604.01.01-E.5159).

**Results**

Out of 12 patients, 11 were male and one female. The mean age was 61 (47–68). ECOG performance status was 0–2. Out of 12 patients, 11 patients’ sera showed positive cytotoxic activity over cut off value, in NeuGcGM expressing L1210 cell line. Mean progression free survival (PFS) for these patients was 13.8 (8–21) months. One patient’s PFS lasted as long as 21 months whose initial stage was IIIB.

In 9 patients, cytotoxic activity was progressively increased at consecutive vaccinations on the 3rd, 6th, 9th and 12th months (Figure 3).

One patient’s cytotoxic activity (patient 1) dropped
below cut off limits at the 12th month of vaccination. Another patient’s (patient 3) cytotoxic test became negative at the 9th month. Further vaccinations are being administered to these two patients whose disease clinical status is still in stable condition.

Out of the 12 patients 3 patients progressed well. Patient 5 at the 21st month from the start of racotumomab, patient 8 at the 9th month and patient 9 at the 11th month. The rest of the patients are stable without progression. All 3 patients who progressed showed a reduced percentage of cell kill in their last cytotoxic test assays. Patient 5 was not able to continue to racotumomab beyond 15th application although he was in complete clinical remission until then.

Best cytotoxic responses to patients’ immune sera, treated with racotumomab occurred at the 6th and 9th month of vaccination, approximately between 9 to 12 intradermal application of racotumomab (Figure 4 and Figure 5).

In summary, 75% of the vaccinated patients showed progressively increased cell kill in their consecutive assays and all these patients are clinically, in stable condition. Two patients who progressed showed reduced cell kill in their consecutive assays despite continuing vaccination. However, one of these patients progressed after discontinuation of racotumab. This patient’s cytotoxic test results were positive until 15th month when he decided not to take the vaccine.

Electron microscopic photographs of cellular swelling and the formation of membrane gaps due to the loss of cell membrane integrity in the culture is a proof of oncotic necrosis induced by Anti-NeuGcGM3 antibody (Figure 6)

A specific anti-N-glycolyl-GM3 response was observed, and weak antibody titers detected to mainly IgG and, few to IgM almost in all patients (10 out of 12). However, only 2 patients’ antibody titers elevated beyond cut off levels which was considered to be positive (patients 5 and 11). The best antibody response was reached at the 8th and 10th dose of vaccination. These patients also showed parallel cell kill result at the cell culture. However apart from these two patients, there were no correlation between cytotoxic tests and antibody response and clinical status of patients in others.

We also looked for potential predictive value of NeuGcGM3 expression intensity in the tumor tissue for efficacy outcome. However, there was no significant
Cytolytic tests with hyperimmune patient sera is a good prognostic tool in racotumomab immunotherapy in advanced non-small cell lung cancer.

Figure 4. Day 0 Cytotoxicity: Analysis of cytotoxicity of patient 12 before the initiation of racotumomab (Day 0) is 16.93%. Separated dead cells (red dots) and living cells (blue cells) on forward scatter (FSC) vs propidium iodide (PI) gate. 10000 events on the gate of FSC vs. PI.

Note: P1: Percentage of death cells in rate of singlet cells; P2: Rate of singlet cells.

Figure 5. Month 9 Cytotoxicity analysis of cytotoxicity of patient 12 at the 9th month of vaccination, 68.8% cytotoxic activity is demonstrated. Separated dead cells (green dots) and living cells (red cells) on forward scatter (FSC) vs propidium iodide (PI) gate. 10000 events on the gate of FSC vs. PI.

Note: P1: Rate of singlet cells. P2: Death cells percentage in rate of singlet cells.

Figure 6. Oncotic necrosis process: Scanning electron microscopy (A) Oncotic necrosis process with hyperimmune sera in L1210 cell culture (B) Preimmune sera-negative control.
difference in between IHC staining intensities with 14F monoclonal antibody in terms of cytotoxicity results.

L1210 cells in 100 mL RPMI 1640 culture medium supplemented with 1% FCS were incubated with patients’ sera for 2 h at 37°C, washed three times with PBS, and then fixed with 3.2% glutaraldehyde in 0.1 mol/L sodium phosphate buffer (pH 7.4) for 1 h. These cells were postfixed for 1 h in 1% OsO4, washed three times with PBS, and dehydrated in ethyl alcohol. Cells were mounted onto a metallic stub and coated (Quorum Q150R) with 5 nm thick gold before analyzed by Zeiss Sigma 500. (9 Eylül University Institutes of Biomedicine and Genom).

Discussion

According to the idiotypic network theory proposed by Jerne, it is possible to induce an immune response against an antigenic epitope using anti-idiotype antibody. This is due to the fact that these antigens have the capacity of mimicking the original antigen. Anti-idiotype antibodies (anti-id abs) see other antibodies as the antigen and bind to it. They are antibodies to idiotopes, located in the antigen binding site of another antibody. These anti-id abs can act as surrogates of the original antigen. Modulation of immune response by these anti-id abs, attracted the attention of researchers for the development of anti-id vaccines against tumor associated antigens. Hernandez et al. demonstrated that, patients vaccinated with racotumomab produced antibodies to NeuGcGm3. When immunized patients’ sera exposed to tumor cells in the culture expressing NeuGcGm3, majority of the cells were killed. According to authors, this cytotoxic effect was different from apoptosis because caspases were not involved. There were no complement activity and the process was fast with the destruction of cytoskeleton. They termed this process as oncolysis.

NeuGcGM3 antibodies had been monitored which showed increasing titers with successive doses in the patient with survival benefit. Peak antibody levels were reached after the 6th or 7th dose of vaccination and remained stable for several months. They also performed cytotoxic tests with immunized patients’ sera in L1210 cell lines expressing NeuGcGM3 ganglioside and demonstrated a profound cell kill which they termed as oncolysis. However, these tests and tumor tissue ganglioside expression were not used as biomarkers neither to predict treatment outcomes nor for the selection of the right patient who will benefit most from the vaccine. In most immunotherapy clinical trials using vaccines, response rates as to RECIST criteria are lower and delayed, compare to chemotherapy. Because clinical responses are not reflected to survival, many studies with vaccines prefer to consider overall survival and progression free survival benefits as primary end points. However, in order to understand lack of benefit and quit unnecessary treatment, time lapse is too long, when overall survival (OS) was considered as primary end point. This issue is especially important in countries, where reimbursement of racotumomab are not currently available by the social security systems. Cytotoxic tests are easy to perform and may be used to select beneficiary patients during vaccination process. Since the most cytotoxic responses are achieved by the 9th application of racotumomab, termination of the treatment may be considered in non-responders beyond this point. In patients with positive cytotoxic tests, vaccination must continue beyond 15th application to maintain the benefit.

Antibody monitoring is not an easy or a practical test for widescale use. It is difficult to perform and in our experience, serum IgM and IgG titers mostly did not correlate with cytotoxic test results.

Conclusion

Although one may argue about the positive selection bias of our patients for the cytotoxic tests (stable or responder patients to chemo, and selection of the patients with positive reaction to 14F7 antibody in the tumor tissue), we can claim that cytotoxic test results predict prognosis. When the percentage of cell kill progressively increases in L1210 cell culture in consecutive assays with immune patient’s sera we can predict a better outcome, looking to the longer PFS of these patients. This study meets the primary goal
of the investigation which was to investigate the value of cytotoxic test as a biomarker for treatment outcome. Our observation confirmed that, consistently higher cytotoxicity rates in the cell culture correlated with better progression free survivals of the patients who are on racotumomab maintenance.

In our assay, anti-body responses have been weak and have not been correlated with cytotoxic tests and clinical status, apart from aforementioned two patients. This may well be due to undetected technical reasons and currently under investigation in cooperation with Cuban and Argentinian scientists who are pioneers in the development of anti-NueGcGM3 treatment strategies and conductors of Phase I-III clinical trials with racotumomab. In this study, we could not assess the potential predictive value of NeuGcGM3 expression in the tumor tissue for efficacy outcome, since there was no significant difference in between IHC staining intensities with 14F monoclonal antibody in terms of cytotoxicity results. However, the issue of NeuGcGM3 expression in the tumor tissue as a biomarker deserves further investigation.

**Authors’ contributions**

N Uskent is the main researcher who planned, conducted and coordinated the study. NM Mandel, R Uslu, A Yazar, are investigators from other universities who had contributed with their patients’ sera and provided information of their patients’ clinical status. Z Gulbas and B Berk conducted the cytotoxic tests whereas G Baloglu performed ELISA tests for NeuGcGM3 antibodies. H Baloglu performed NeuGcGM3 expression in the tumor tissue.

**Acknowledgments**

We would like to thank to assistant researcher Sevde Nur Biltekin who conducted the confirmatory cytotoxicity tests at MEDIPOl University and molecular biologist Arzu Dincer who established outstanding coordination between centers. Electron microscopic photographs of antibody induced apoptosis in L1210 cell culture were performed at 9 EYLUL University Institutes of Biomedicine and Genom.

**Conflict of interest**

The authors declare no potential conflict of interest with respect to the research, authorship, and/or publication of this article.

**References**


