USE OF THE EXTRACT OBTAINED FROM THE OLEA EUROPAEA LEAVES IN THE TREATMENT OF GliO-BLASTOMA

Figure 1

Abstract: Invention relates to the use of Olea europaea in cancer stem cell positive Glioblastoma tumors that are treated with temozolomide.

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USE OF THE EXTRACT OBTAINED FROM THE OLEA EUROPAEA
LEAVES IN THE TREATMENT OF GLIOBLASTOMA

DESCRIPTION

Technical Field
Present invention relates to the use of the extract of the leaves of Olea europaea (OLE) in the treatment of Glioblastoma (GBM) tumors which are characterized by cancer stem cells positivity.

Present invention especially relates to the activity of OLE on the tumor tissues detected to contain cancer stem cell among the GBM tumor tissues obtained from the surgery of the patients bearing different clinical properties.

State of the Art
GBM (Glioblastoma), also known as Glioblastoma Multiforme, is the most common primer malign brain tumor among all brain tumors. This tumor is a malignant tumor, a cancer, deriving from the glial supportive tissues in star shape found in the brain, in other words from the astrocytes.

Life time of patients with Glioblastoma (GBM) is limited to 12-15 months after its diagnosis. Despite all of the developments in the technical and pharmacological field in last 25 years, the additional gain of life time has been merely 3 months. Recurrence is unavoidable after the primary surgery, radiotherapy and chemotherapy (TMZ) (%85). Temozolomide (TMZ) has been commonly used nowadays in the treatment of patients with Glioblastoma. The activity of TMZ, however, differs according to the gene expression 06-methyl guanine-DNA methyl transferase (MGMT) of the patient. Moreover, presence of the stem cells gives rise to GBM tumors to resist against radiotherapy and pro-apoptotic chemotherapy and lead to aggressiveness of the disease. Therefore the desired success has not yet been achieved in the treatment of patients with GBM by the treatment methods of our day.

Cancer stem cells responsible from tumor development and invasion show resistance against the medicaments that are currently used to treat glioblastoma, aiming to inhibit the growth factors, intracellular signal pathways or invasion processes. TMZ has been commonly used nowadays in the treatment of patients with GBM.
One of the PCT applications encountered throughout the literature search is WO201 3086308 (A1). Said invention relates to use of TMS in the treatment of brain cancer.

One of these patent applications is US201 204091 4 (A1). Said invention relates to the use of TMZ in the treatment of Glioblastoma.

The activity of TMZ however, shows differences according the MGMT gene expression of the patient. Further, combination therapy of TMZ with radiotherapy has major cytotoxic effects for the patient. Even if the treatment methods having such cytotoxic effects are effective in eliminating the tumor to a great extent, they may give rise to negative side effects for the patient. Recent studies show that the patients, who are specified to contain cancer stem cells in their tumors, are likely to be much less responsive to the TMZ treatment.

New treatment methods in recent years for various cancer types frequently make use of plants. As a result of evaluating the mechanism of action by scientific studies of the traditionally used herbal products by people, therapeutic agents such as vinblastine, vincristin, topotecan, irinotecan, etoposide and pacitaxel have been developed and started to be used in the treatment protocol.

Olive (Olea europaea) is a strong antioxidant which is traditionally used in treatment of various diseases in European and Mediterranean countries. Extracts derived from the olive leaves contain vast amounts of bioactive components such as secoiridoid, tripertene and flavonoids. Most important bioactive component contained by said extract is oleuropein which is a secoiridoid. To this day the antitumoral properties of OLE has been studied in various studies, and it has been shown that this extract induces an antiproliferative and apoptic effect in evaluations on cancers of leukemia, colon, melanoma and breast.

One of these applications is JP2008273938 (A). The invention relates to the use of olive leaves in the types of cancers such as the gastric cancer, colon cancer.

Another of these patent applications is CN1 01607068 (A1). Said invention relates to use of olein in the treatment of liver cancer.

Hence, due to the inconveniences mentioned above and insufficiency of the solutions to this matter, it is obvious that there is a need for a development in the technical field regarding the treatment of GBM's containing cancer stem cells.
Object of the Invention

Present invention relates to the use of OLE in the treatment of cancer stem cells which meets the above mentioned requirements, eliminates the drawbacks and brings in further advantages.

Aim of the present invention is especially the activity of OLE on the tumorous tissues detected to contain cancer stem cell among the GBM tumorous tissues obtained from the surgery of the patients bearing different clinical properties.

An object of the invention is, thanks to its activity on cancer stem cells, to contribute in generating supportive protocols in the treatment of patients with GBM resisting against medicaments, in order to reduce the resistance against chemotherapeutic agents.

A similar object of the invention is to enhance the quality of life of the patient by reducing the possible side effects, owing to the fact that the OLE has a lower cytotoxic effect on normal cells of the patient and that it allows lower doses of TMZ to be used, which otherwise would have a high cytotoxic effect.

In order to achieve aforementioned objects Olea europaea is used in cancer stem cells positive Glioblastoma tumors that are treated with temozolomide.

Structural and characteristic features and the advantages of the present invention will be understood more clearly in light of the detailed description with reference to the following figures and thus the evaluation should be carried out considering these figures and the detailed description.

Drawings Which Will Be Helpful in Understanding the Invention

Figure 1: Isolation of cancer stem cells from GBM tumors
A: 2nd day of the culture
B: 7th day of the culture
C: Magnetic separation of cancer stem cells
D: Early phase neurosphere formation in cancer stem cells (X40)

Figure 2: is the designation of the positiveness of CD133 and Nestin which are precursors of cancer stem cells via flow cytometry technique.

The drawings are not necessarily to be scaled and the details that are not needed to comprehend present invention may have been neglected. Additionally, at least substantially
identical elements or at least the elements substantially of identical functions, are shown by
same numbers.

**Detailed Description of the Invention**

In this detailed description, preferred embodiments of the use of OLE in the treatment of
cancer stem cells of the present invention is merely disclosed with the intent of providing a
clearer understanding of the subject and in a non-limitative manner.

The invention relates to the antitumoral activity of the olive (*Olea europaea*) on the cancer
stem cell positive Glioblastoma tumors treated with temozolomode (TMZ).

**Preparation of olive (*Olea europaea*) for use in GBM cancer stem cells mentioned in the
present invention:**

- Olive leaves are picked in the pruning season, February, in the region of Bahkesir,
  Edremit, washed and overshaded to dry,
  (Thus, since *Olea europaea* burgeons freshly in February, the risk to contain hazardous
  chemicals such as pesticides and herbicides on the pruned branches is eliminated).
  - Leaves that are kept in dark and at room temperature for a month are then grinded in
    a mill and kept in 80% ethanol at room temperature,
  - In order to prevent the oleuropein, the active ingredient of *Olea europaea*, from
    being spoilt, ethanol of the ethanol extract is turned into an extract powder by being
    evaporated at low pressure and temperature not exceeding 55°C in a Spray Dryer
    system,
  - Thus obtained olein (OLE) is used by dissolving in sterile dH₂O.

Figure 1 shows the isolation of cancer stem cells from GBM tumors. In order to prepare the
primary cell cultures belonging to the tumoral tissues obtained from the patients, a part of the
Glioblastoma tumors obtained from the patients via surgical intervention was sent to the
Medical Biology AD Cell Culture Laboratories in PBS containing 5% antibiotics. Here, after
being transferred into PBS containing 2% antibiotics and washed for several times, tissue
samples were divided into small particles in the order of a pinhole with the aid of scalpel and
forceps and transferred in to cell culture plates of 25cm². Cells, after being supplied with the
growth medium are cultured without being displaced for 7 days. On day 7, culture plates
were controlled under inverted microscope and the growth medium of the primary culture
plates were replaced with serum free DMEM F12 growth medium containing 5µg/ml Heparin,
50 ng/ml Epidermal growth factor, 20 ng/ml fibroblast growth factor, %2 B27, 10ng/ml
Leukemia inhibitor factor and 1% antibiotic/antimycotic solution. When the cells grew as much as to cover the culture plate, on the cells that were picked up by applying trypsin, GBM stem cells were separated via magnetic separation of the CD133 (+) cells.

In order to control the purity of the cancer stem cells; by using anti-human CD133 and anti-human Nestin-1 monoclonal antibodies (Figure 2), flow cytometry analysis of the cells were performed. For this, CD133+ cells that were adhered to the surface of the culture plates belonging to the early stage passages of the cells separated via magnetic nanoparticles, were picked from the surface by 0.25% trypsin-EDTA. And they were suspended according to the kit procedure. Then the cells were treated with fluorescein isothiocynate (FITC) specific to CD133 and Nestin, phycoerythrin (PE)-conjugate monoclonal antibodies and suitable isotype controls. In order to clear the possible artifacts, washing was performed with PBS containing 0.1% sodium azide and cell suspension was scanned by FACS Calibur Flow Cytometry (BD Sciences). Analysis of obtained data were performed by BD CellQuestTM software.

Further, for validation of the cancer stem cell, by performing RNA isolation, expression of the genes Vimentin, Integrin, CD44 and OCT4 which are specific to stem cells, was controlled by RT-PCR method.

Table 1. Control of the other cancer stem cell precursors

<table>
<thead>
<tr>
<th></th>
<th>P1</th>
<th>P2</th>
<th>P3</th>
<th>P4</th>
<th>P5</th>
<th>P6</th>
<th>P7</th>
<th>P8</th>
<th>P9</th>
<th>P10</th>
<th>10% Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>FNGA</td>
<td>1.8453</td>
<td>8694.5281</td>
<td>0.0021</td>
<td>9.3401</td>
<td>0.0201</td>
<td>58.8356</td>
<td>0.164008</td>
<td>7943.5487</td>
<td>0.2958</td>
<td>941.4037</td>
<td>0.0003</td>
</tr>
<tr>
<td>VDM</td>
<td>0.2133</td>
<td>5999.3855</td>
<td>0.5386</td>
<td>840.2825</td>
<td>0.5380</td>
<td>81.4903</td>
<td>2.431403</td>
<td>3713.2846</td>
<td>0.0421</td>
<td>44.8335</td>
<td>0.0006</td>
</tr>
<tr>
<td>CD44</td>
<td>0.7845</td>
<td>1434.8236</td>
<td>0.2357</td>
<td>440.2735</td>
<td>0.2150</td>
<td>41.9154</td>
<td>0.205223</td>
<td>557.9492</td>
<td>0.1158</td>
<td>211.8158</td>
<td>0.0005</td>
</tr>
<tr>
<td>OCT4</td>
<td>3.7580</td>
<td>660.1576</td>
<td>0.0028</td>
<td>0.4900</td>
<td>0.0200</td>
<td>7.0200</td>
<td>0.335335</td>
<td>57.1494</td>
<td>0.4673</td>
<td>83.6716</td>
<td>0.0016</td>
</tr>
</tbody>
</table>

* tumorous tissue of the patient
* epileptic tissues were used as non-tumorous control tissues

As mentioned in Table 1, tissues, the amount of the expression of the cancer stem cell precursors of which are at least 2 times higher compared to non-tumorous tissues are considered to be cancer stem cell positive.

To this end, isolation of the RNA from the CD133+ cells obtained and reproduced from primer tumors was performed by mRNA isolation kit specific to cell culture. Quantification and qualification controls of the RNAs were performed by nanodrop. For the complementary DNA (cDNA) synthesis reaction, final RNA concentration was adjusted at least to 1-5 µg.
cDNA synthesis was performed from the RNAs by using Transcriptor First Strand cDNA Synthesis Kit. From obtained cDNAs, by using specific primers belonging to *Vimentin*, *Integrin*, *CD44* and *OCT-4* genes which are cancer stem cell markers and primers specific to *B-actin* gene as control, aimed levels of mRNA expression was determined. Suitable PCR mixture prepared for the expression was reproduced by using LC 480 Real Time device according to PCR protocol specific to primers. Changes in the mRNA expressions were relatively determined by performing ΔΔCT method for data analysis.

In order to investigate the effect of the OLE and TMZ doses on miRNA expressions in the GBM stem cells (Table 2), by using standard commercial kit methods, miRNA isolation and cDNA synthesis were performed from GBM stem cells.

Table 2. Changes in the miRNA expressions in GBM stem cells after OLE and TMZ treatment

<table>
<thead>
<tr>
<th>Treatment</th>
<th>mir-181b</th>
<th>miR-153</th>
<th>miR-145</th>
<th>miR-137</th>
<th>let-7d</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated</td>
<td>0.412367</td>
<td>0.000379</td>
<td>0.007922</td>
<td>0.000518</td>
<td>0.026388</td>
</tr>
<tr>
<td>1 mg/ml OLE</td>
<td>0.544876</td>
<td>0.004796</td>
<td>0.080772</td>
<td>0.004796</td>
<td>0.17776</td>
</tr>
<tr>
<td>Amount of change in the expression (fold)</td>
<td>1.3213</td>
<td>12.682</td>
<td>1.965</td>
<td>9.2663</td>
<td>6.7365</td>
</tr>
<tr>
<td>95 % CI</td>
<td>0.13, 2.51</td>
<td>0.0001, 42.64</td>
<td>37.00</td>
<td>32.83</td>
<td></td>
</tr>
<tr>
<td>* P value</td>
<td>0.6212</td>
<td>0.1375</td>
<td>0.1876</td>
<td>0.1754</td>
<td>0.6828</td>
</tr>
<tr>
<td>2 mg/ml OLE</td>
<td>0.713013</td>
<td>0.011296</td>
<td>0.034722</td>
<td>0.011296</td>
<td>0.136408</td>
</tr>
<tr>
<td>Amount of change in the expression (fold)</td>
<td>1.7291</td>
<td>29.8157</td>
<td>4.3832</td>
<td>21.8264</td>
<td>5.1694</td>
</tr>
<tr>
<td>95 % CI</td>
<td>0.05, 3.41</td>
<td>0.0001, 90.40</td>
<td>17.36</td>
<td>27.89</td>
<td></td>
</tr>
<tr>
<td>* P value</td>
<td>0.3557</td>
<td>0.0088</td>
<td>0.4323</td>
<td>0.0155</td>
<td>0.3847</td>
</tr>
<tr>
<td>450 μM TMZ</td>
<td>0.088388</td>
<td>0.016289</td>
<td>0.080214</td>
<td>0.016289</td>
<td>0.46974</td>
</tr>
<tr>
<td>Amount of change in the expression (fold)</td>
<td>0.2143</td>
<td>42.9921</td>
<td>10.1261</td>
<td>31.4721</td>
<td>1.7802</td>
</tr>
<tr>
<td>95 % CI</td>
<td>0.0001, 132.10</td>
<td>0.0001, 59.91</td>
<td>7.18</td>
<td>27.89</td>
<td></td>
</tr>
<tr>
<td>* P value</td>
<td>0.4428</td>
<td>0.0118</td>
<td>0.2239</td>
<td>0.0155</td>
<td>0.2864</td>
</tr>
<tr>
<td>1 mg/ml OLE + 450 μM TMZ</td>
<td>9.958994</td>
<td>0.028676</td>
<td>0.056563</td>
<td>0.018149</td>
<td>0.004588</td>
</tr>
<tr>
<td>Amount of change in the expression (fold)</td>
<td>24.1508</td>
<td>75.6884</td>
<td>7.1404</td>
<td>35.0660</td>
<td>0.1739</td>
</tr>
<tr>
<td>95 % CI</td>
<td>105.23</td>
<td>0.0001, 258.73</td>
<td>26.75</td>
<td>113.99</td>
<td></td>
</tr>
<tr>
<td>* P value</td>
<td>0.0842</td>
<td>0.1774</td>
<td>0.3209</td>
<td>0.0323</td>
<td>0.1360</td>
</tr>
<tr>
<td>2 mg/ml OLE + 450 μM TMZ</td>
<td>1.503161</td>
<td>0.031034</td>
<td>0.583984</td>
<td>0.017027</td>
<td>0.14376</td>
</tr>
<tr>
<td>Amount of change in the expression (fold)</td>
<td>3.6452</td>
<td>81.9118</td>
<td>73.7208</td>
<td>32.8996</td>
<td>5.4490</td>
</tr>
<tr>
<td>95 % CI</td>
<td>0.72, 6.57</td>
<td>0.0001, 257.85</td>
<td>259.87</td>
<td>111.76</td>
<td></td>
</tr>
<tr>
<td>* P value</td>
<td>0.0227</td>
<td>0.0438</td>
<td>0.0005</td>
<td>0.0146</td>
<td>0.3762</td>
</tr>
</tbody>
</table>

From the obtained cDNAs; in the study performed with T98G cells showing the synergistic effect of OLE with TMZ (1), by using specific primers belonging to miRNAs determined to bear significant changes in expression thereof via OLE treatment, the level of miRNA
expression was determined. For each miRNA expression analysis, expression analysis of a housekeeping gene was performed as well. Further, for control of the RT-PCR reaction, The miRNA Reverse Transcription Control Assay (miRTC) and Positive PCR Control Assays (PPC) control primers were used. Suitable PCR mixture prepared for the expression was reproduced by using LC 480 Real Time device according to PCR protocol specific to primers. Changes in the miRNA expressions were relatively determined by performing ΔΔCT method for data analysis.

Following conclusions were reached according to obtained findings:

1) OLE is effective on the cancer stem cell positive GBM tumors.

2) OLE of the present invention shows cytotoxic effect on GBM stem cells.

3) In the previous study (1), cytotoxic activity of the lymphocyte cells of OLE was determined to be much less as compared to tumor cells. Said study (1) showing the activity of OLE on the GBM cells was performed on purchased T98G cell line. Obtained findings show the activity of OLE on the tumoral tissues detected to contain cancer stem cell among the GBM tumoral tissues obtained from the surgery of the patients bearing different clinical properties.

4) Changes in the miRNA expression levels in GBM stem cells caused by OLE show in vitro that, the use of olenin with TMZ leads to increase in the activity of TMZ, and that by this means, it is possible to reduce possible side effects by reducing the effective dose of TMZ.

5) Enhancing the quality of life of the patient by reducing the possible side effects, owing to the fact that the OLE has a lower cytotoxic effect on normal cells of the patient and that it allows lower doses of TMZ to be used, which otherwise would have a high cytotoxic effect.

5) OLE shows synergistic effect with TMZ on GBM stem cells.
REFERENCES

1. Invention is characterized by the use of *Olea europaea* in the cancer stem cell positive Glioblastoma tumors that are treated with temozolomide.
Figure 2
INTERNATIONAL SEARCH REPORT

A. CLASSIFICATION OF SUBJECT MATTER
INV. A61 K36/63 A61P35/O0
ADD.

B. FIELDS SEARCHED
Minimum documentation searched (classification system followed by classification symbols)
A61K A51 P

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronical database consulted during the international search (name of database and, where practicable, search terms used)
EPO-Internal, BIOSIS, COMPENDEX, EMBASE, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

<table>
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<th>Category</th>
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<th>Relevant to claim No.</th>
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<tr>
<td>Y</td>
<td>* pp. 1832-1833, bridging *</td>
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* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier application or patent published on or after the international filing date
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T Special categories of cited documents:

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"Z" document member of the same patent family

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Laffargue-Haak, T

See patent family annex.
<table>
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<th>Citation of document, with indication, where appropriate, of the relevant passages</th>
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<tr>
<td>Y</td>
<td>GULCIN TEZCAN ET AL: &quot;miRNA Expression Pattern Modulates Temozolomide Response in GBM Tumors with Cancer Stem Cells&quot;. CELLULAR AND MOLECULAR NEUROBIOLOGY, vol. 34, no. 5, 2 April 2014 (2014-04-02), pages 679-692, XP055218216, ISSN: 0272-4340, DOI: 10.1007/S10571-014-0050-0 abstract * p. 690, col. 1, last *, figure 5</td>
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<td>TEZCAN GULCIN ET AL: &quot;Olea europaea leaf extract improves the treatment response of GBM stem cells by modulating miRNA expression&quot;. AMERICAN JOURNAL OF CANCER RESEARCH, vol. 4, no. 5, 6 September 2014 (2014-09-06), pages 572-590, XP55218202, ISSN: 2156-6976 the whole document</td>
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