Lung Cancer 104 (2017) 16–23

Contents lists available at ScienceDirect

Lung Cancer

journal homepage: www.elsevier.com/locate/lungcan

Heterogeneity of circulating tumor cells (CTCs) in patients with recurrent small cell lung cancer (SCLC) treated with pazopanib☆

I. Messaritakis[a], E. Politaki[a], M. Plataki[a], V. Karavassilis[c], N. Kentepozidis[d], F. Koinis[a,b], E. Samantas[e], V. Georgoulias[a,b,*], A. Kotsakis[a,b]

[a] Laboratory of Tumor Cell Biology, School of Medicine, University of Crete, Greece
[b] Department of Medical Oncology, University General Hospital of Heraklion, Crete, Greece
[c] Department of Medical Oncology, "Papageorgiou" General Hospital, Thessaloniki, Greece
[d] Department of Medical Oncology, 251 Air Forces General Hospital, Athens, Greece
[e] First Department of Medical Oncology, "Agioi Anargyroi" Anticancer Hospital, Athens, Greece

ARTICLE INFO

Article history:
Received 26 October 2016
Received in revised form 7 December 2016
Accepted 13 December 2016

Keywords:
TTF-1
CD56
VEGFR2
Immunofluorescence
CellSearch
SCLC
CTCs
Pazopanib

ABSTRACT

Objectives: To investigate the effect of pazopanib on different CTCs subpopulations in patients with recurrent SCLC and evaluate their clinical relevance.
Methods: Peripheral blood was obtained before the administration of pazopanib (n = 56 patients), after the first cycle (n = 35 patients) and at disease progression (n = 45 patients). CTCs were detected by CellSearch and double immunofluorescent staining using antibodies against the cytokeratins (CK), TTF-1, CD56 and VEGFR2.
Results: Before treatment, CTCs could be detected in 50% of patients by CellSearch; phenotypic characterization of CTCs demonstrated that 50%, 46.6% and 27.6% of patients had $CD45^-/TTF1^+$, $CD45^-/CD56^+$ and $TTF\text{-}1^+/CD56^+$ CTCs, respectively. Additionally, 59% of CTCs were $TTF\text{-}1^+/VEGFR2^+$ and 53% $CK^+/VEGFR2^+$. One pazopanib cycle resulted to a significant decrease of the number of CTCs (CellSearch: $p = 0.043$) and $CK^+/VEGFR2^+$ cells ($p = 0.027$). At the time of PD, both the total number of CTCs ($p = 0.027$) and the number of the different subpopulations were significantly increased compared to post-1st cycle values; this increased CTCs number was associated with a significant increase of $TTF\text{-}1^+/VEGFR2^+$ ($p = 0.028$) and $CK^+/VEGFR2^+$ CTCs ($p = 0.018$). In multivariate analysis, only the number of CTCs as assessed by CellSearch after one treatment cycle was significantly associated with OS (HR: 0.21; $p = 0.005$).
Conclusions: Pazopanib has a significant effect on different subpopulations of CTCs in patients with relapsed SCLC; the detection of $VEGFR2^+$ CTCs during treatment could be a surrogate marker associated with resistance to pazopanib.

1. Introduction

SCLC is a chemo- and radio-sensitive tumor and almost 70–80% of the patients experience an objective clinical response with front-line treatment. However, the disease prognosis is still poor, since after an initial response, almost all patients will relapse and eventually succumb to their disease [1–3]. This is mainly due to the rapid hematogenous tumor dissemination where one of the mechanisms demonstrated is the relation between density of microvessels, expression of vascular endothelial growth factor (VEGF) and development of metastases and poor prognosis [4,5]. Pazopanib is a small molecule with a tyrosine multi-kinase inhibitor activity, targeting the VEGFR-1, VEGFR-2, VEGFR-3, PDGF and c-KIT [6]. In preclinical models, pazopanib inhibited phosphorylation of VEGFR-2, c-KIT and PDGFR-β receptors in a dose-dependent manner [7], and has shown substantial activity in renal cell cancer and sarcomas [8,9].

Detection of circulating tumor cells (CTCs) in peripheral blood could serve as a prognostic and predictive tool in various cancers, including SCLC [10–15]. In SCLC, CTC numbers, circulating tumor microemboli (CTM) and apoptotic CTCs have been emerged as independent prognostic factors [12,16–21]. Detection of CTCs using

☆ Preliminary results presented as a poster presentation at the 1st ACTC, September 26–29 2012, Athens, Greece, at the 10th ISMRC, March 19–21 2016 in Hamburg, Germany and at the ASCO, June 3–7 2016, Chicago, IL, USA.

* Correspondence to: Laboratory of Tumor Cell Biology, University of Crete, School of Medicine, Stavrakia and Voutes, 71100 Heraklion, Crete, Greece.
E-mail addresses: georgoul@med.uoc.gr, georgoul@uoc.gr (V. Georgoulias).

http://dx.doi.org/10.1016/j.lungcan.2016.12.008
0169-5002/

cytology has the advantage that cells maintain their morphology, permitting their phenotypic and molecular characterization. SCLC is characterized by expression of various neuroendocrine peptides, such as CD56 (NCAM), and the Transcription Thyroid Factor-1 (TTF-1) [22–31]. Based on that expression, we have recently reported that at least three different subpopulations of CTCs (TTF-1^+/$CD45^-$, $CD56^+$/$CD45^-$ and TTF-1^+/$CD56^+$) could be detected in patients with newly diagnosed SCLC; these were maintained during front-line tretament with etoposide/platinum regimens although treatment resulted in a significant decrease of their absolute number [21].

The Lung Cancer Working Group of the Hellenic Oncology Research Group (HORG) conducted a multicenter, phase II trial of second-line pazopanib in patients with recurrent/refractory SCLC. In order to investigate whether changes of specific CTC subpopulations could serve as biomarker associated with an anti-angiogenic treatment, we prospectively evaluated the various CTC subpopulations of patients with SCLC treated with 2nd line pazopanib. The hypothesis behind this research was to investigate whether changes of specific CTC subpopulations could serve as biomarker associated with an anti-angiogenic treatment.

2. Patients and methods

2.1. Patients' eligibility criteria

Patients aged >18 years old with cytologically/histologically documented SCLC and disease relapse/progression after the standard front-line chemotherapy (etoposide/platinum) or chemo-radiation were enrolled in the study. Patients had a Performance Status (ECOG) 0–2, measurable disease (RECIST criteria 1.1), adequate organ function tests and an expectancy of life of $\geq$3 months. Brain metastases were allowed, provided that they were previously treated by radiotherapy and the patient was neurologically stable. Exclusion criteria among the standard for phase II studies were (i) gastrointestinal abnormalities that increase the risk for gastrointestinal bleeding (ii) presence of uncontrolled infection, (iii) corrected QT-interval (QTc) >480 msecs (using Bazett's formula), (iv) history of active uncontrolled coronary heart disease and congestive heart failure and (v) poorly controlled hypertension (Koinis F et al.; Manuscript submitted). The study has been approved by the Ethics and Scientific Committees of the participating institutions, the Hellenic Drug Organization (EOF) and the National Ethics Committee (EED). All patients signed a written informed consent for their participation.

2.2. Patient samples and cytospin preparation

Peripheral blood (20 ml in EDTA and 7.5 ml in CellSave preservative tubes; Raritan, USA) was obtained before pazopanib initiation (baseline; n = 58), after one-cycle (post-1st cycle; n = 37) and on clinical or radiological disease progression (PD; n = 48) (Supplementary Fig. 1). All blood samples were obtained at the middle-of-vein puncture after discarding the first 5 ml of blood to avoid contamination with skin epithelial cells.

Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll–Hypaque density gradient (d = 1077 g/mL; Sigma-Aldrich Chemie GmbH, Germany) centrifugation at 1800 rpm for 30 min at ambient temperature. Aliquots of 5×10^5 PBMCs were cytocentrifuged at 2000 rpm for 2 min on glass microscope slides. Cytospins were dried and stored at −80 °C until use. Two slides (10×10^5 PBMCs) from each patient were analyzed at each timepoint.

2.3. Detection of CTCs using the CellSearch platform (CS)

For CTCs enumeration using the CellSearch, blood samples maintained at ambient temperature and processed within 72 h. The CellSearch (Veridex LCC, Raritan, NJ) assay was performed according to the manufacturer's instructions. CTC morphology was confirmed in all cases and analysis was performed with the CellTracks Analyser II. Results are expressed as number of CTCs/7.5 ml blood.

2.4. Double immunofluorescence assay

PBMCs cytospins were fixed with ice cold aceton:methanol 9:1 (v/v) for 20 min. Incubation period for all primary/secondary antibodies was 1 h. CTCs were double stained with rabbit anti-CD45 (Common Leukocyte Antigen; Santa Cruz, USA) and either anti-TTF-1 (DAKO, Agilent Technologies, Denmark) or anti-CD56 (NCAM; ThermoFisher Scientific, USA) antibodies in order to exclude hematopoietic cells expressing these proteins. The presence of double stained TTF-1/CD56 CTCs was also investigated. Zenon technology (FITC-conjugated IgG1 antibody; Molecular Probes, Invitrogen, USA) was used for TTF-1detection. CD56 was labelled with Alexa-555 or Alexa-488 (Molecular Probes) and CD45 was detected using an anti-rabbit antibody labelled with Alexa-555. Moreover, cytospins were double stained with the mouse anti-TTF-1 or anti-A45-B/B3 (anti-cytokeratins 8/18/19; Micromet, Germany) and the rabbit anti-VEFGR2 (Cell Signaling, USA) antibodies. For this, cytospins were fixed with 3% paraformaldehyde for 30 min. Cell membrane permeabilization was performed with 0.5% Triton for 10 min, followed by overnight incubation with 1% BSA/PBS. Subsequently, cytospins were stained with TTF-1 or A45-B/B3 and VEGFR2 with the corresponding primary and secondary antibodies for 1 h, each. TTF-1 and A45-B/B3 were labelled with an anti-mouse Alexa-488 and VEGFR2 with an anti-rabbit Alexa-555 antibody. The omission of the first antibody has been used as negative control. DAPI-antifade reagent (Molecular Probes) was added for nuclear staining. Slides were analyzed using a fluorescent microscope (Leica DM 2500, Heidelberg, Germany). Results are expressed as number of CTCs/10^6 PBMCs.

2.5. Determination of the sensitivity of double immunofluorescence

The human SCLC cell line, H209 (ATCC® HTB-172) obtained from the American Type Culture Collection (Manassas, USA) and used as positive control for both TTF-1 and CD56 (Messaritakis I et al.; Phenotypic heterogeneity of circulating tumor cells in patients with small cell lung cancer (SCLC). Manuscript submitted). H209 were cultured in RPMI 1640 (Gibco BRL Life Technologies, NY), supplemented with 10% FBS (Gibco), 2 mM L-glutamine (Gibco), 10 mM Hepes (Gibco), 1 mM sodium pyruvate (Gibco), 1.5 g/L $NaHCO_3$ (Sigma-Aldrich), 4.5 g/L glucose (Sigma-Aldrich) and 50 mg/ml penicillin/streptomycin (Gibco). Cells were maintained in a humidified atmosphere, 5% CO_2, 37 °C.

To determine the sensitivity of the method, H209 cells were spiked in peripheral blood of healthy individuals, and cytospins were prepared as per patients' samples. All experiments were performed during the logarithmic growth phase of the cells.

2.6. Study design and statistics

This is a correlative translational research study evaluating the effect of pazopanib on the detection of CTC subpopulations in patients with recurrent/refractory or resistant SCLC treated in the second-line setting with pazopanib in the context of an open label, multicenter, non-randomized, Phase II study (NCT01713296).