Application of array-Comparative Genomic Hybridization analysis in immune-virotherapy approach

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ABSTRACT

Introduction: oncololytic adenoviruses (OAds), viruses constructed to replicate in tumor cells, improve the outcome of cancer therapy in some cases, such as sarcomas. However, the molecular heterogeneity of tumors requires specific and personalized cancer treatments in order to set up more adequate and effective therapies.
Methods: by using the array Comparative Genomic Hybridization (array-CGH), a molecular approach method, we aimed to identify chromosomal aberrations or Copy Number Variants (CNVs) in three different tumor cell lines: HCT116, SW872 and A2058 selected for their Coxsackievirus and Adenovirus receptor (CAR) expression profile.
Results: the cells showed several duplications of genes involved in replicative Adenovirus cycle (binding, internalization, escape) in the core transport, and in the escape of the viral DNA from the capsid.
Conclusion: in this study, our aim was to identify chromosomal alterations in genes involved in the OAd replication cycle process. Array-CGH method could be useful to design a platform for a screening analysis in order to identify mutations that can contribute to oncolytic virotherapy approach generating a personalized strategy for tumor suppression.

INTRODUCTION

The discovery of new technologies led to development of more sensitive diagnostic methods and new types of cancer treatment, such as virotherapy (1) in order to overcome some of the radiotherapy and chemotherapy limitations.
Actually, virotherapy, especially in a combinatorial approach, could be tested in some types of tumor, such as sarcomas. It is important to understand the "actors" involved in the pathways that promote cell proliferation in order to establish an effective and personalized therapy, like the chromosomal aberrations involved in intra-patient intra-tumor heterogeneity (2).
A better understanding of the molecular mechanisms underlying the replication of oncolytic vectors (OVs), engineered to preferentially replicate in tumor cells, could be useful to improve the efficacy of OV-based therapy. In addition, the genetically unstable nature of tumors could be responsible for resistance to any therapeutic approaches (3). Therefore, we supposed that genetic screening of several tumor cell lines could help to design and optimize new therapeutic approaches. In fact, it was reported that tumor cells possess high mutation and chromosome rearrangement rates (4) giving, in some cases, growth advantage and high plasticity to modulate gene expression and signal transduction pathways (5). We focused our attention on the evaluation of chromosomal aberrations or copy number variations (CNVs) in order to identify gene alterations involved in the adenovirus replicative cycle that may constitute a prognostic factor for virotherapy approaches. Array-Comparative Genomic Hybridization (array-CGH) methodology allows an analysis of the

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whole genome and a rapid detection of the genomic imbalances involved in the etiology of several genetic disorders and cancers (6,7) and could be helpful to improve the understanding of tumor genetics.

METHODS

Cell lines and culture conditions

Twelve cell lines are used in this study. Human liposarcoma SW872 (ATCC® HTB-92™), human melanoma A2058 (ATCC® CRL-11147™), muscle rhabdomyosarcoma RD (ATCC® CCL136™), human vulva leiomyosarcoma SK-LMS-1 (ATCC® HTB-88™), human synovial sarcoma SW982 (ATCC® HTB-93™), and human fibrosarcoma HT1080 (ATCC® CCL-121™) cell lines were purchased from American Type Culture Collection (ATCC). Human lung carcinoma A549 (DSMZ® ACC-107™), human colon carcinoma HCT116 (DSMZ no. ACC-581), human chronic myelogenous leukemia K-562 (DSMZ no. ACC-10), and human acute monocytic leukemia THP-1 (DSMZ no. ACC-16) cell lines were purchased from Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH - German Collection of Microorganisms and Cell Cultures (DSMZ). Human colorectal adenocarcinoma Caco-2 (ECACC 86010202) was purchased from European Collection of Authenticated Cell Cultures (ECACC) and human ovarian carcinoma HEY from Tebu-bio no. CLU302-A, Magenta, Italy. All tumor cell lines were grown according to manufacturers' procedures.

Flow cytometry

The Coxsackievirus and Adenovirus receptor (CAR) expression profile was performed using the protocol already reported. Briefly, the fluorochrome-conjugated monoclonal antibody (mAb) anti FITC CAR (Santa Cruz Biotechnology, Inc.; Dallas, Texas, U.S.A.) and labeled isotype control (IgG2a) were used. After vigorous washing, cells were transferred to fluorescent-activated cell sorter (FACS) tubes and analyzed with flow cytometer (Gallios, Beckman Coulter, Miami, USA). Samples were acquired in duplicates, and analyzed with Flow Jo software (Tree Star, Ashland, OR, USA) (8).

Viability assay

The cell lines cultures were infected with Ad5D24, vector carrier of the d24 deletion in E1 (9) and, at the highest infectivity conditions (100 virus particles per cell-vp/cell), an extensive cytopathic effect (>90%) was evident at microscopy. The cell viability has been determined by using Cell Proliferation Colorimetric Assay MTS according to the manufacturer's protocol (Cell Titer 96 Aqueous One Solution Cell Proliferation Assay; Promega, Nacka, Sweden). The absorbance was measured at 490 nm on Varioskan Flash Multimode Reader (Thermo Scientific, Carlsbad, CA, USA) and spectrophotometric data were acquired. The experiments were independently performed three times and each experiment contained triple replicates. Uninfected cells were used as control.

Array - Comparative Genomic Hybridization

Array-CGH analysis was performed by using the Agilent Human Genome CGH Microarray 4x 180K (Agilent Technologies, Santa Clara, CA, USA), according to manufacturer's procedure (10–12).
The array-CGH platform we used, is a dual color array containing 170,334 60-mer oligonucleotide probes with a spatial resolution of 13 kb that covers the whole genome. Microarrays were scanned on an Agilent G2600D scanner and image files were quantified using Agilent's Feature Extraction software (V11.5.1.1); data were visualized with Agilent's Genomic Work Bench Standard Edition (V7.0.4.0). Results are interpreted as $\log^2$ ratio of test versus control.

Real Time Polymerase Chain Reaction (PCR)

Real Time PCR analysis was performed to assess the amplification and quantification of the number copy of *E4* gene after infection of cell lines cultures with Ad5D24. Primer pairs for *E4* (13) and for *GAPDH* genes were used for analysis according to a validate PCR reaction protocol (14); in particular, to generate a normalizing amplicon, the *GAPDH* gene was used (15). Data represent the mean (SD) of two independent experiments, each performed in duplicate, and are presented by comparison to control (uninfected cells). *E4* gene expression resulted higher in SW872 compared to A2058 and HCT116, respectively.

Statistical analysis

GraphPad Prism 6 (GraphPad Software, Inc., La Jolla, CA, USA) was used to perform data analysis by using one-way or two-way ANOVA with Tukey's post test, considering as variables the time and the different cell lines. Results are presented as mean (SD). A detailed description of the statistical methods used to analyze the data of each experiment can be found in the figures' captions.

RESULTS

Expression profile of CAR and viability test

In order to assess whether the tumor cell lines were susceptible to infection by the oncolytic adenovirus Ad5D24, it was necessary to evaluate the CAR expression. We selected the 3 cell lines, (HCT116, SW872,A2058) that showed the highest CAR profile among the 12 tumor cell lines, by using flow cytometry based on the average of geometric mean fluorescence intensity (gMFI) by comparison with the other cell lines (figure 1). Data represent mean (SD) of triplicate experiments. The cytopathic effect (CPE) is around 100% after infection, with a concentration of 100 vp/cell of Ad5D24 virus at several time points (3, 6, and 7 days

respectively for HCT116, SW872, and A2058 cell lines) (figure 2 A, B, C).

Residual cell viability was evaluated by MTS assay showing greater oncolytic activity on HCT116 [2.6136 (2)] cell line compared to SW872 [4.3705 (0.8)] and to A2058 [1.0067 (0.4)] cell lines (figure 3 A, B, C) at 100 vp/cell. Data represent mean (SD) of duplicate experiments . All data achieved statistical significance ($p \leq 0.0001$), using one-way ANOVA test. Figure 3 reports the significance of the concentration of 100 vp/cell of Ad5D24, corresponding to the CPE.

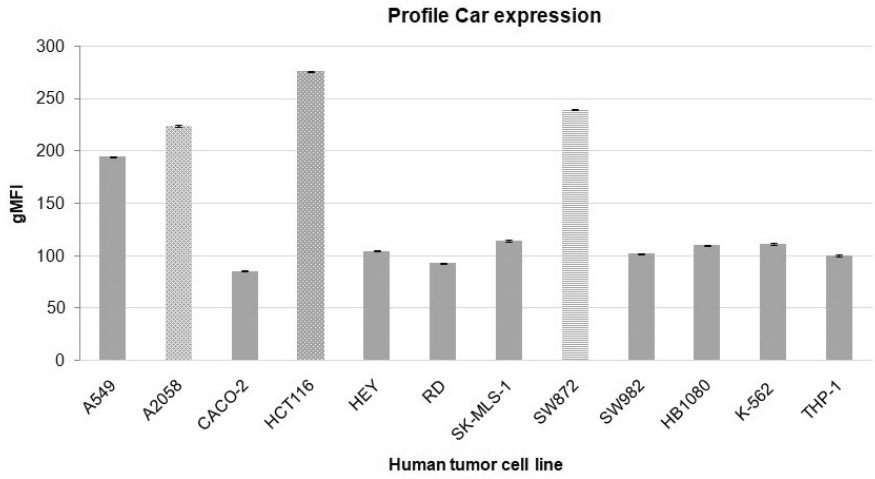

Figure 1
Profile of CAR receptor expression in different tumor cell lines. Geometric mean fluorescence intensity (gMFI) of CAR receptor expression is shown for each cell line. The data are shown as mean (SD), n = 3).

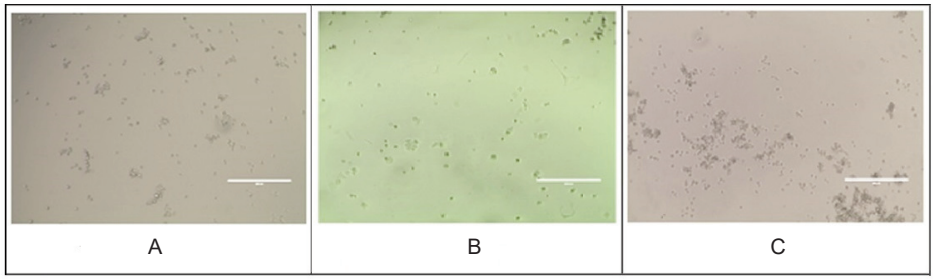

Figure 2
Scan Optical Microscope images of cytopathic effect of different cell lines. Cytopathic effect was obtained with 100 vp/cell of Ad5D24 at 3 days, 6 days and 7 days in HCT116 (A), SW872 (B) and A2058 (C), respectively. Scale bar indicates 400 µm.

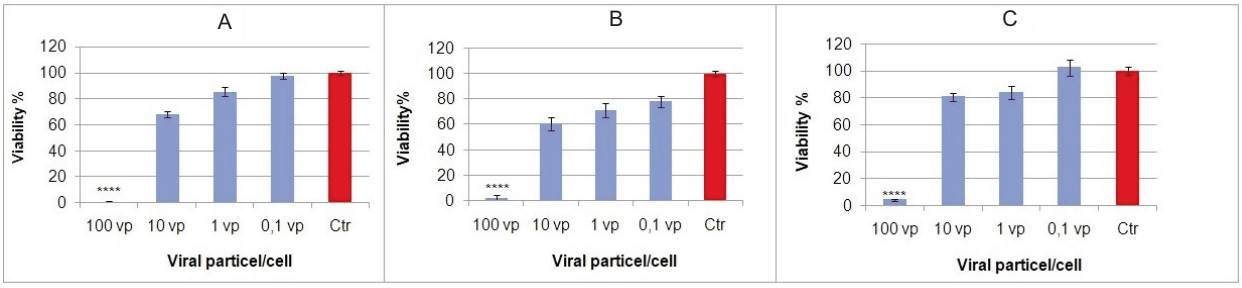

Figure 3
Vitality test MTS. Test was perfomed on HCT116 (A), SW872 (B) and A2058 (C) infected with different concentration of Ad5D24 (0.1, 1, 10 and 100 vp/cell) and it was performed when the cytopathic effect was achieved at a concentration of 100 vp/cell of Ad5D24. Uninfected cells were used as controls (Ctr). The data are shown as mean (SD), n = 2). Significance was assessed using one-way ANOVA test; $p \leq 0.0001$

Table 1
Chromosomal aberrations of replication cycle of adenovirus. The alterations reported are: duplication (Dup) and amplification (Amp).

Adenovirus pathway trafficking	Gene	Chromosomal region	Alteration	Cell line
Absorption	*CXADR* (Coxsackie Virus And Adenovirus Receptor)	21q21.1	Dup	SW872
Absorption/ internalization	*ITGB3* (Integrin, Beta 3)	17q21.32	Dup	A2058 HCT116 SW872
Entry	*CLTC* (Clathrin, Heavy Chain)	17q23.1	Dup	A2058 HCT116 SW872
Entry	*CLINT1* (ClathrinInteractor 1)	5q33.3	Amp	SW872
Entry	*CLTA* (Clathrin, Light Chain A)	9p13	Dup	SW872
Entry	*CAV1* (Caveolin 1)	7q31.1	Dup	A2058 SW872
Entry	*CAV2* (Caveolin 2)	7q31.1	Dup	A2058 SW872
Escape	*ATP6V1B2* (ATPase, H+ Transporting, Lysosomal 56/58kDa, V1 Subunit B2)	8p21.3	Dup	SW872
Escape	*ATP6V1H* (ATPase, H+ Transporting, Lysosomal 50/57kDa, V1 Subunit H)	8q11.2	Dup	A2058 SW872
Escape	*ATP6V1C1* (ATPase, H+ Transporting, Lysosomal 42kDa, V1 Subunit C1)	8q22.3	Dup	A2058 HCT116 SW872
Traslocation	*DYNLRB1* (Dynein, Light Chain, Roadblock-Type 1)	20q11.21	Dup	SW872
Traslocation	*DYNC2H1* (Dynein, Cytoplasmic 2, Heavy Chain 1)	11q21-q22.1	Dup	SW872
Traslocation	*DYNLL2* (Dynein, Light Chain, LC8-Type 2)	17q22	Dup	A2058 HCT116 SW872
Traslocation	*KLC1* (Kinesin LightChain1)	14q32.33	Dup	SW872
Nuclear Import	*NUP214* Nucleoporin 214kDa)	9q34.1	Dup	SW872
Nuclear Import	*NUP205* (Nucleoporin 205kDa)	7q33	Dup	A2058 SW872
E1A	*E2F1* (E2F TranscriptionFactor 1)	20q11.2	Dup	SW872
E4	*GABPA* (GA Binding Protein Transcription Factor, Alpha Subunit 60kDa)	21q21.3	Dup	SW872
E1A/E1B	*TP53* (TumorProtein P53)	17p13.1	Dup	A2058

Array - Comparative Genomic Hybridization analysis and *E4* gene expression

Array-CGH method performed on the 3 selected cell lines, revealed different chromosomal aberrations in genes involved in the replicative cycle of Adenovirus.
Table 1 reports the gene mutations involved in the cell membrane adsorption, internalization, lysis of endosomes, translocation, nuclear import and the early genes that will lead to virus replication. To increase the array-CGH result and to verify the pathway of oncolytic adenovirus infection, we analyzed the expression of *E4* gene for its role in viral replication. *E4* expression profile was determined by Real Time PCR in HCT116, SW872 and A2058 cell lines at different time points: 2, 24, 48 and 72 hours after Adenovirus infection (figure 4). Data represent the mean (SD) of two independent experiments, each performed in duplicate, and are presented by comparison to control (uninfected cells). *E4* gene expression resulted higher in SW872 compared to A2058 and HCT116, respectively. Significance was assessed using two-way ANOVA test ($p \leq 0.01$ and $p \leq 0.0001$).

DISCUSSION

In order to improve the application of OAds in immune-viral therapy, it is necessary to understand and clarify the molecular pathways responsible of infection and replication in tumor cell lines.
The first step of the main adenovirus intracellular trafficking pathway is the vector binding to receptor at the cell surface (16). CAR, the main receptor involved in Adenovirus infection, is expressed by several normal cell types but its expression level varies highly in tumor cells (17). However, Bagheri et al. (18) observed that a limited virus activity is due to a reduced CAR expression. Among 12 different tumor cell lines, we selected 3 cell lines (HCT116, SW872 and A2058) expressing the highest virus internalization efficiency. Our data, obtained by flow cytometry analysis, showed that the CAR expression profile is higher in the HCT116 compared to SW872 and A2058 cell lines; the results are well correlated to the HCT116 cell viability (100% of cell death in 3 days).
Since the array-CGH platform used in this pilot study is designed for human genes, we were not able to observe mutations in *E4* gene, but on the other hand we could indirectly identify possible gene alterations, in selected tumor cell lines, involved in pathways of OV proteins in order to investigate the efficacy of virus replication. To our knowledge, no data have been reported until now regarding the identification of genetic alterations by using array-CGH analysis in tumor cell lines treated with onco-virotherapy. In this study, we observed that SW872 cells showed several duplications of genes involved in the replicative Adenovirus cycle (binding, internalization, escape) in the core transport, and in the escape of the viral DNA from the capsid.
Our aim was to analyze gene alterations involved in the nucleus entry that allow the transcription of early stage genes, necessary to induce the entry in the S phase, to escape the antiviral defense systems and to synthesize proteins essential for viral DNA replication, such as E2F, GABPA and TP53 (19,20). These genomic regions showed duplications in SW872 cell line.

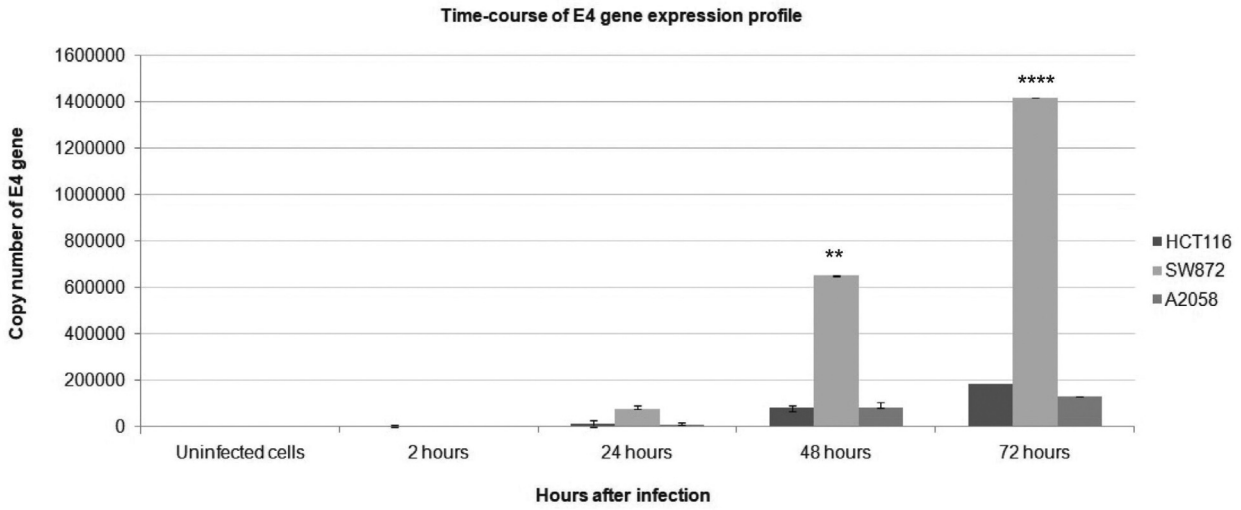

Figure 4
Time-course of E4 gene expression profile performed by Real Time PCR in HCT116, SW872 and A2058 cell lines at 2, 24, 48 and 72 hours after Ad5D24 infection. SW872 cell line showed a greater E4 gene expression compared to HCT116 and A2058, respectively. Data analysis was performed by using two-way ANOVA with Tukey's post test considering as variables the time and the different cell lines. The data are shown as the mean (SD), n = 2). Significance was assessed using two-way ANOVA test; $p \leq 0.01$ $p \leq 0.0001$.

Of particular interest is the *GABPA* gene duplication. This gene codes a transcription factor necessary for the expression of adenoviral *E4* gene (20). Proteins expressed from the *E4* region are implicated in the regulation of a set of functions during productive infection including transcription, viral DNA replication, RNA splicing and processing, late protein synthesis and the transition from the early to late stages of infection (21). Moreover, *E4* gene products target cellular regulators of cell signaling and DNA repair contributing to cell transformation and oncogenicity. The *E4* promoter is activated very early after infection by the E1a transcriptional activator protein; at later times transcription declines due to repression by the *E2a* gene product and feedback inhibition of E1a-mediated transactivation by the E4orf4 protein; in fact many E4 open reading frame (orfs) produce multifunctional viral regulators. Mutations in *E4* regions could results in defects in viral DNA replication, accumulation of late viral messages and proteins, virus particle assembly and shut-off of host protein synthesis. Then, to increase the significance of the array-CGH results and to better clarify the possible correlation between *GABPA* and *E4* gene expression, we performed a Real Time PCR. The results suggested that the duplication of the *GABPA* gene could play a primary role in adenoviral replication; in fact, the SW872 cell line showed a significant increase ($p < 0.05$) of *E4* gene copy number compared to the others cell lines, suggesting that genetic alterations might be responsible for the different efficacy of OAds. On the other hand, we observed the highest oncolytic activity in HCT116 cell line compared to the others, where the *GABPA* gene duplication is not present, suggesting that other factors could be involved in the regulation of oncolytic activity in the above mentioned cell. Furthermore, additional studies are necessary to better understand which other factors are involved in the *E4* pathway.

Therefore, the presence of gene duplications, as *GABPA* gene in SW872 cell line, could give a positive advantage in replicative Adenovirus cycle (binding, internalization, escape), in the core transport, and in the escape of the viral DNA from the capsid.

In conclusion, our data suggested that genetic mutations in the viral replication cycle of Adenovirus could contribute to explain differences of the oncolytic effect/efficacy of OAds.

Moreover, future studies on genetic alterations in genes involved in cell-cycle of the host cell and its checkpoints could be effective to realize a suitable platform design for array-CGH analysis and to combine this diagnostic method to the immune-virotherapy in order to set up a personalized therapy for tumor eradication.

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