



Auditorium CNR - Pisa

Friday, July 3, 2009

ABSTRACT BOOK



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Auditorium CNR - Pisa
Friday, July 3, 2009

Programme

- 8.15 *Welcome and registration*
- 8.30 *Official opening*
Presidente CNR di Pisa
Claudio Montani
Magnifico Rettore dell'Università di Pisa
Marco Pasquali
Assessore al Diritto alla Salute Regione Toscana
Enrico Rossi
- 9.00 ONCO-HAEMATOLOGY
- BCR-ABL
Persio Dello Sbarba (Firenze)
- PNH
Rosario Notaro (CRL, Firenze), Cristina Nativi (Firenze)
- AML
Francesco Mannelli (Firenze)
- HCV-lymphoma
Anna Linda Zignego (Firenze)
- COMMENTARY
Mario Petrini (Pisa)
- 10.10 CLINICAL CANCER RESEARCH - I
- Clinical cancer trials
Luca Boni (CCSC, Firenze)
- Phase I clinical trials
Romano Danesi (Pisa), Enrico Desideri (Firenze)
- Compliance with standards of care
Eugenio Paci (Firenze)
- COMMENTARY
Paolo Vineis (London)
- 11.00 *Coffee break*

- 11.30 GENOME INSTABILITY AND CANCER
Cell cycle checkpoints
Antonio Musio (Pisa)
GGT and cancer progression
Alfonso Pompella (Pisa)
COMMENTARY
Silvestro Conticello (CRL, Firenze)
- 12.10 CANCER CELL BIOLOGY
Signalling through MAP kinases
Mario Chiariello (CRL, Siena)
ETV4 and prostate cancer
Maria De Angioletti (CRL, Firenze)
Hedgehog signalling in stem cells and cancer
Barbara Stecca (CRL, Firenze)
COMMENTARY
Pier Paolo Pandolfi (Boston)
- 13.10 *Lunch break*
- 14.15 CLINICAL CANCER RESEARCH - II
Targeting mitochondria in glial tumors
Leonardo Rossi (Pisa)
Mutations of the EGF receptor
Fulvio Basolo (Pisa)
Intelligent drugs
Paolo Bruzzi (Genova)
Intelligent surgery
Paolo Bechi (Firenze)
Intelligent radiation
Luigi Pirtoli (Siena)
COMMENTARY
Bob Pinedo (Amsterdam)
- 15.45 COMMUNICATION WITH PATIENTS WITH CANCER
Manuel Katz (Firenze)
A protocol and role playing
(Pietro Manno, Enrico Ruggini)
Questions/discussion
- 16.30 *Tea break*
- 17.00 GUEST LECTURE
Regulation of the p53 network
Gianni Del Sal (Trieste)
- 18.00 Adjournment

Epidermal Growth Factor Receptor and K-RAS Mutations in Lung Adenocarcinoma

Fulvio Basolo

Department of Surgery, University of Pisa

Targeting the epidermal growth factor receptor has played a central role in advanced non-small cell lung cancer research, treatment, and patient outcomes over the last several years; however a number of questions about this approach remain to be addressed. Through the Istituto Toscano Tumori and the Italian Association of Women Against Lung Cancer Project, we collected 411 lung adenocarcinomas from several clinical centers in Tuscany. Mutations were assessed by sequencing exons 18-21 of the epidermal growth factor receptor gene, and by restriction fragment length polymorphism analysis of codons 12 and 13 of the K-RAS gene. Epidermal growth factor receptor mutations (12.6%) were more frequently observed in females ($p < 0.0001$), in non-smokers ($p = 0.005$), and in the presence of bronchioloalveolar features ($p = 0.0004$). K-RAS mutations (17.9%) were more frequent in males ($p = 0.0007$) and were associated with smoking habits ($p = 0.005$). Epidermal growth factor receptor and K-RAS mutations were mutually exclusive ($p = 0.001$). We focused on 21 female patients with advanced/metastatic lung adenocarcinoma who received gefitinib 250 mg/day (expanded access) or erlotinib 150 mg/die as second/third-line therapy; partial response was associated with classic epidermal growth factor receptor mutations ($p = 0.006$) and with a non-smoking history ($p = 0.02$). None of the female patients with partial response and /or stable disease showed K-RAS alterations.

Although the data obtained in our study have yet to be analyzed and confirmed with a larger number of patients treated with tyrosine kinase inhibitors, they should provide useful information for targeted therapy, in particular for non-smoking female lung cancer patients.

Intelligent surgery

Paolo Bechi

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Intelligent surgery is the one that causes the least possibility of collateral effects avoiding damage to anything else but the target.

Firstly, surgery causes collateral effects because the approach to the target takes place through superficial tissues, which must be discontinued and cut.

Secondly, the need of oncologic radicality implies to taking away of potentially non-affected tissues, which will only be discovered to be affected, or not, at the histopathological exam (for example the draining nodes).

Therefore, we are dealing with the toracoscopic/laparoscopic and robotic surgery and the sentinel lymph-node evaluation.

Laparoscopic surgery has been the most important surgery novelty for the past 100 years. The advantages of laparoscopic surgery are numerous: not only on account of the small incision but also owing to a reduced haemorrhage, less pain-hypothermia-dehydration, reduced exposure of internal organs to external contaminants, with the consequential reduced risk of infections, shorter recovery time and faster return to an everyday living. Most of the operations are currently performed with this technique and its indications are becoming wider and wider. However, an evaluation of its oncological results is now available only as far as colorectal cancer is concerned.

The COLOR (Colon cancer Laparoscopic or Open Resection) trial show comparable results between the two groups of 500 patients each, as far as disease free survival is concerned. Studies concerning the laparoscopic procedures for gastric, oesophageal, pancreatic and ovarian cancers are under way and probably few years will be needed before results from the oncological standpoint will be available.

Robotic surgery represents a development of laparoscopic surgery. The main advantages are represented by: 3-dimensional view, more accurate movements, tremor filtration and wristed instrumentation, which allow a better dissection and less damage to the surrounding tissues. All these advantages result in a further reduction of collateral effects. Another advantage is represented by the possibility of operations in remote sites. The disadvantages are mainly due to high cost of instrumentation, long surgical times and length of surgical training. Oncologic results are available only for poststatic carcinoma. For the treatment of this neoplasm, robotic surgery is becoming the gold standard. In general surgery, although at present, the data from controlled studies are not available, the most appropriate indications for robotic surgery seem to be represented by the radical lymphadenectomy in gastric, pancreatic, and rectal cancer. Future applications of robotics are unpredictable due to the easily predictable developments in technology.

The third aspect of intelligent surgery is linked to the possibility of less invasive "tailored" operations. One of the main aspects is represented by the study of the sentinel lymph node. This method is now well consolidated in melanoma and breast cancer. Its application in gastric and colon cancer could allow less invasive operations with the possibility of significant lower morbidity and mortality. However, when applied to these neoplasms the methodology is still nothing more than an experimental, also by reason of the more unpredictable arrangements of the draining lymphatic system in the stomach and colon when compared to breast.

In a series of 855 patients consecutively operated upon, for colon-rectal cancer, we studied 57 patients (colon cancer cT1cN0cM0, and cT2cN0cM0), 18 underwent open surgery and 39 laparoscopic surgery. All the subjects underwent standard surgical resection with an en-bloc regional lymphadenectomy. A lymph node mapping was performed "in-vivo" in patients who underwent open surgery and with an "ex-vivo" technique in the laparoscopic group with a detection rate of 96.5%, a sensitivity of 100%.

We are now evaluating autofluorescence techniques for the detection of metastatic lymph nodes in colon cancer but the experience is absolutely preliminary.

An example of independent clinical research: the interim results of the STAR (Studio Terapia Adiuvante Retto)-01 randomized phase III trial.

Luca Boni

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Introduction: The CTCC operates to promote multi-institutional independent research through the development, management, and reporting of hypothesis-driven clinical trials. STAR-01 is one of the trials in charge to CTCC and the preliminary results of this study are the object of the present report.

Background of the trial: Oxaliplatin (OXA) enhances the efficacy of fluorouracil (FU)-based chemotherapy in colon cancer¹. STAR-01, a randomized phase III trial, investigated the effect of adding OXA to preoperative (preop) FU-based pelvic chemoradiation (CRT) in patients (pts) with locally-advanced rectal cancer^{2,3}.

Methods: Eligibility required a resectable, biopsy-proven rectal adenocarcinoma within 12 cm from the anal verge with radiological evidence of perirectal fat or lymphnode involvement. Randomization was between infused FU (225 mg/msq/day) concomitant to external-beam pelvic radiation (50.4 Gy in 28 daily fractions) (arm A) or the same regimen + weekly OXA (60 mg/msq x 6) (Arm B). Total Mesorectal Excision surgery was scheduled 6-8 weeks after completing CRT. Overall survival was the primary end-point. The protocol-planned analysis of local tumor response to preop treatment (secondary end-point) is described.

Results: 747 pts (752 registrations with 5 pts randomized twice) from 41 Italian centers were randomized between 12/2003 and 8/2008 (arm A/B: 379/368). Pre-treatment characteristics were similar in arm A/B: median age 63/62 years; male to female ratio 2.2:1/2.0:1; median T distance from anal verge 6 cm; T4 20/17%, N+ 63/67%. Overall grade 3-4 toxicity rates on treated pts were 8% (arm A) and 24% (arm B) with diarrhoea being the most common side effect in both arms. 364/353 pts (arm A/B) had surgery at a median interval from the end of CRT of 52/53 days, 15 pts in each arm were not operated (progression 7, death 5, other/unknown 18). Pathologic response data analyzed on all the randomized population (intention-to-treat principle) are reported in the table.

Conclusions: The addition of weekly OXA to standard FU-based preop CRT significantly increases toxicity without affecting local tumor response. Longer follow-up is needed to assess the impact on other efficacy end-points.

	ARM A N=379	ARM B N=368	p value
Pathological stage			
T0N0	16.1%	16.3%	0.938
N1-2	25	27	0.498
M1	3.2%	0.8%	0.022

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Intelligent drugs

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All drugs are developed hoping that they will show some degree of intelligence, that is, that they will do more good than harm. Specifically, all anticancer drugs that were developed during the last 40 years tried to take advantage of some differences between normal and cancer cells, in order to selectively kill (or stop the growth of) cancer cells without causing serious adverse effects in the patient. The peculiar characteristic of cancer cells most often exploited by past anticancer drugs was their high rate of proliferation, and not surprisingly many of the toxic effects of these drugs affected cell systems with rapid turnover, such as the hematopoietic cells and the mucosal cells. However, other important toxic effects, such as cardiotoxicity and neurotoxicity, were seen that could not be directly related to the mechanisms by which cytotoxic drugs kill rapidly proliferating cells. Another example is the development of the antiestrogen Tamoxifen, which showed a striking efficacy against hormone-dependent breast cancer, but also a paradoxical estrogen-like effect in some organs with resulting toxicity and adverse events.

In this perspective, the last generation of anticancer drugs, also known as molecularly targeted drugs, does not represent a conceptual revolution. It is simply, once more, the application of scientific and technical advances to the development of treatments. Yet, in recent years, the progress in the understanding of the processes underlying cancer development and progression represents a true quantum leap, and is allowing the development of drugs with completely novel mechanisms of action. The expectation of a dramatic advance in the efficacy of anticancer therapies, with little or no toxicity, has been only partially fulfilled: these drugs are showing clinical efficacies that in few instances are paradigm-changing, but are more often moderate, while their toxicity is not always negligible. More important, the molecular markers that were expected to predict their efficacy, sometimes proved to be the wrong ones. Overall, it appears that a mechanistic approach is appropriate and necessary in the design and preclinical development of these new drugs, but that the traditional empiricist approach is still needed in the assessment of their clinical value. Due to the peculiar properties of these drugs, the clinical trials required for this assessment pose unique methodological and statistical challenges, such as, for instance a) innovative endpoints and designs for phase II trials, b) extensive reliance on subgroup analyses in phase III trials, and c) Bayesian designs in trials in rare molecular subgroups.

Signaling through MAP kinases

Mario Chiariello

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Activating mutations and overexpression of several human oncogenes stimulate the enzymatic activity of mitogen-activated protein kinases (MAP kinases). Moreover, the activity of some of these kinases is found highly increased in several types of human tumors or is required for the therapeutic effect of antitumoral drugs. The wide contribution of MAP kinases to virtually any aspect of normal and aberrant cell proliferation therefore makes them central targets to achieve a successful cancer therapy. Still, beside being able to develop new drugs with low or null secondary effects, the possibility of a successful use of MAP kinase inhibitory compounds in the cure of cancer will be determined by our ability to decipher the complexity of each MAP kinase signaling route. In this frame, while there is a well-developed body of knowledge that suggests different MAP kinases as critical regulators of cell proliferation and human cancer, it is still entirely unclear how the specific Erk8 member of this family participates to these processes.

Erk8, the last identified member of the MAP kinase family, is controlled by activated oncogenes and DNA-damage, and regulates the activity and stability of proteins necessary for cellular proliferation. Also, modifications in its levels has been recently associated to breast cancer progression and we have shown that three human oncogenes, RET/PTC3, RET/MEN2B and bcr/abl, are able to activate Erk8 and mediate their downstream signaling. These data therefore suggest a role for this kinase in normal and aberrant cell proliferation and point to modulation of its activity as a powerful tool to treat human cancer.

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3. Catania A. Iavarone C., Carlomagno M.S. and Chiariello M. Selective transcription and cellular proliferation induced by PDGF require histone deacetylase activity (2006). *Biochem. Biophys. Res. Commun.* 343: 544-554.
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Phase I clinical trials

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A phase I clinical trial is the first stage of drug testing in human subjects. A small group of healthy or ill volunteers are selected on the basis of clinical selection criteria and administered the test drug. The choice of healthy subject or patient is based on the risk category to which the drug belongs to. The main goal of this phase is the assessment of drug safety. However, modern phase I clinical trials are integrated with the activity of clinical laboratories committed to 1) identification of target disease and optimal drug combinations to be administered to the patient, 2) development of pharmacokinetic and pharmacodynamic modeling, 3) identification of biomarkers to monitor drug response and toxicity, 4) selection of predictive genetic factors for the stratification of patients based on their likelihood of responding to a drug or suffering from toxicity, 5) clinical application of advanced bio-imaging and immunological monitoring of drug effects in the subject, and 6) planning sample size (biostatistics) and developing mathematical models for advanced analysis of clinical and laboratory data (bioinformatics).

These trials are often conducted in an inpatient clinic and include dose-ranging design for the identification of the appropriate dose for therapeutic use. However, the previous concept of dose escalation up to intolerable toxicity is being replaced by the dose inducing the optimal biologic effect, a strategy particularly important for optimal development of anticancer agents. Finally, pharmacokinetics [1] and pharmacogenetics [2-4] are gaining momentum in the novel generation of early clinical trials for their ability to identify drug distribution profiles and genetic signatures potentially useful for the identification and appropriate stratification of the "fit" patient.

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Role of ETV4 Expression in prostate cancer

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Prostate cancer is the most commonly diagnosed cancer in elderly men in Western countries. Recently, chromosomal translocations that juxtaposes the promoter of a gene highly expressed in prostate to the coding sequence of one member of the ETS gene family (*ERG*, *ETV1*, *ETV4*, *ETV5*) have been found: these result in the over-expression of a specific ETS protein. We have focused on investigating the pathogenic role of the over-expression of *ETV4* gene that has been found juxtaposed to the promoter of *TMPRSS2*, *KLK2* and *CANT1*. We have studied the levels of expression of *ETV4* in few human prostate cell lines: *ETV4* expression was not detectable in LnCap and V-Cap whereas it was present in PC3 and Du145 cell lines. In order to investigate the role of *ETV4* expression in prostate cancer, we have used a doxycycline-inducible expression of short hairpin (sh) RNAs against *ETV4*: after induction, the growth of DU145 cell line transduced with the shRNA containing vector was about 50% of that of DU145 transduced with an empty vector. In addition, we have observed that the reduction of *ETV4* expression strongly impairs the ability to form colonies in soft agar ($\geq 65\%$ of reduction). *ETV4* shRNA interference has produced similar reduction of cell growth and of the number soft agar colonies of PC3 cell line. Furthermore, the growth of DU145 xenografts are strongly reduced by the induction of *ETV4* shRNA interference. These preliminary experiments show that the expression of *ETV4* is functionally important in a cellular model of prostate cancer; thus, they suggest that *ETV4* expression may play a direct role in the development and progression of a subset of prostate cancers.

Regulation of the p53 network

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After its first description, thirty years ago, as a nuclear protein engaged by the SV40 large T antigen, and the realization that p53 is a powerful tumor suppressor inactivated in about 50% of tumors, the scientific community has invested a formidable amount of research on understanding how the p53 gene and protein are regulated. p53 is a transcription factor acting as a central hub in a molecular network controlling cell proliferation and cell death in response to a plethora of stress stimuli and oncogenic conditions. Stress signals are transduced in a wide array of covalent modifications and protein interactions that modulate either nuclear and cytoplasmic activities of p53. Understanding how these modifications impact on the p53 activity is essential for dissecting both wt and mutant p53 functions. In this context a crucial role is played by phosphorylation-dependent conformational changes caused by the prolyl-isomerase Pin1. This protein, binds and catalyzes cis/trans isomerization of prolines on phosphorylated S/P or T/P motifs in many proteins involved in tumour growth, and is emerging as an important modifier of cell signalling. The impact of this protein on wt and mutant p53 activities as well as the role of new p53 modulators will be discussed.

Hypoxia and glucose shortage modulate Chronic Myeloid Leukaemia cell phenotype and leukaemia stem cell properties

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Background & aim: We previously showed that Chronic Myeloid Leukaemia (CML) stabilized and cloned cell lines are highly heterogeneous populations, where incubation under severe hypoxia selects leukaemia stem cells (LSC) and suppresses non-stem clonogenic progenitors (LPC). Hypoxia also suppresses BCR/Abl expression completely, so that hypoxia-resistant LSC resulted BCR/Abl-independent as for their survival *in vitro* and exhibited complete resistance to imatinib-mesylate (IM), a feature representing a high risk for the establishment of minimal residual disease. What we described is a novel mechanism of refractoriness of LSC to IM which is not limited to a small LSC subset, but is a property of the whole LSC population which is consistently exhibited in response to specific environmental conditions (hypoxia). Such a mechanism is therefore different from the “secondary” resistance to IM developed by mutant CML subclones as a consequence of chronic IM treatment. The aim of the study we present here was to evaluate separately the effects of the shortage of oxygen only (“hypoxia”) or of oxygen and glucose (“ischemia”) on the selection of CML cell subsets, and to undertake the characterization of these effects. Possible long-term outcome of this study is to provide a basis to design therapeutical approaches targeted to hit LSC specifically and thereby to the long-term eradication of leukaemia.

Methods: Hypoxic (0.1% O₂) CML cultures were established with different time-zero cell densities and glucose concentrations and treated or not with IM. BCR/Abl expression was assessed by western blotting. Stem cell potential was determined by the Culture-Repopulating Ability (CRA) assay, where the selection of LSC in primary cultures (LC1) is evaluated following their transfer to non-selective secondary cultures (LC2).

Results: Incubation under severe hypoxia at low time-zero cell density is compatible with a significant expansion of the number of viable cells. However, after 2-3 weeks of incubation, hypoxia causes cell number to return to the time zero level. Glucose was exhausted in culture medium after 2-3 weeks at low and after 1 week at 1-log higher time-zero cell density. Thus, in hypoxia, the kinetics of cell number decrease seems to parallel that of glucose decrease. In normoxia, on the contrary, glucose exhaustion was compatible with the maintenance of relatively high cell concentrations, as shown before with the growth curves. In hypoxia, but not normoxia, BCR/Abl protein was eventually suppressed at either time-zero cell density. In either case, the kinetics of BCR/Abl protein suppression also reflected that of the decrease of glucose concentration. Time-zero glucose concentration was the varied. In high-glucose cultures, the maintenance of relatively high glucose levels throughout the incubation was paralleled by the maintenance of relatively high levels of BCR/Abl protein expression and cell number. At low time-zero glucose, glucose exhaustion at day 6 was followed by the suppression of BCR/Abl and the drop of cell number at day 7. These results indicated that also the kinetics of BCR/Abl protein expression reflectes that of glucose concentration.

Low cell density hypoxic cultures at critical times of incubation were then assayed (CRA) as for the maintenance of LSC. Cell transfer to LC2 from day-14 LC1, upon glucose exhaustion (and BCR/Abl suppression), immediately repopulated LC2. Cell transfer at day 21, following glucose exhaustion (and after BCR/Abl suppression had been consolidated), repopulated LC2 after an 1-week lag. Thus, in low cell density LC1, hypoxia allowed to select, at different incubation times, two different subsets of LSC, characterized by early and late or late only CRA. As summarized above, both these subsets appear to belong to the BCR/Abl-negative fraction of cultured cells.

The effects of IM on early and late LC2 repopulation by cells rescued from LC1 at day 14 were then determined. IM significantly reduced early, but not late, LC2 repopulation. Thus, LSC responsible for late repopulation were refractory to IM.

Conclusions: The use of low cell density cultures allowed to identify two different subsets of hypoxia-resistant LSC characterized by BCR/Abl suppression. One subset, selected when glucose is exhausted but environmental conditions are still merely hypoxic, contains cells closer to the LPC phenotype and immediately recruitable to clonal expansion upon transfer to growth-permissive normoxic cultures. Such a property, apparently due to the prompt rescue of BCR/Abl expression, explains their relative sensitivity to IM. The other subset, arguably on the average more immature, selected under frankly ischemic conditions, contains cells capable to drive late LC2 repopulation only and refractory to IM. Thus, glucose availability in the environment appears to finely regulate the balance between different hypoxia-resistant, BCR/Abl-negative LSC subsets.

**Communication with patients with cancer
A protocol and a role playing**

Manuel Katz

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Il protocollo Spikes per comunicare cattive notizie verrà sinteticamente illustrato. La sua applicazione verrà esemplificata con una simulazione tra un oncologo e un attore professionista cui farà seguito una riflessione sulle competenze necessarie al medico per poter applicare correttamente il protocollo. Verrà presentata letteratura rilevante.

Kinetics of leukemic cells in AML: predictive value

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The prognosis of patients with Acute Myeloid Leukemia (AML) is widely variable. Conventional cytogenetic analysis and gene abnormalities escaping cytogenetic detection (i.e. FLT3, NPM1, CEBPA, MLL) allow the stratification of patients in groups with different probabilities to achieve and maintain complete remission (CR), providing the basis for risk-oriented treatment strategies. However these data are not usually available at the time of starting treatment and induction is delivered regardless of genetic sub-characterization of AML. Therefore risk-oriented treatment concerns post induction phase only.

To predict the individual response in a clinically relevant time, we investigated the potential predictive value of the clearance of peripheral blasts (PBC) during standard induction course in AML by Flow Cytometry (FC) 1, 2 and by RT-PCR for *Wilms Tumor 1* gene expression. Both methods showed excellent correlation with CR achievement and survival indicating that PB may be in equilibrium with BM in each AML patient, and that PBC gives evidence of bone marrow response. Therefore, a major treatment outcome may be predicted very early during the induction therapy of AML patients, thus providing an opportunity to tailor treatment modalities since the outset.

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Cell cycle checkpoint: the role of Claspin gene in genome instability

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It has long been known that cancer is frequently associated with genome instability resulting from cell cycle checkpoint failure. Structural chromosomal rearrangements represent a hallmark of cancer cells. In fact, marker chromosomes, containing complicated rearrangements of several chromosomes, are frequently seen in cancer. The molecular basis for such rearrangements is largely unknown. Fragile sites exhibit several features characteristic of highly unstable and recombinogenic genome regions. It has recently been shown that instability at fragile sites is a hallmark of early precancerous lesions and it is thought that most chromosomal rearrangements in solid tumors originate from fragile sites (Arlt et al., 2006). In spite of extensive analysis of fragile sites, determining the molecular basis for chromosome instability at common fragile sites has been elusive. It has been hypothesised that common fragile sites are regions of DNA whose replication is unusually sensitive to interference and chromosomal gaps and breaks are due to un- or under-replicated DNA (Sbrana et al., 1998) in specific chromosomal regions resulting from stalled forks that escape replication checkpoint (Arlt et al., 2004; Musio et al., 2005; Durkin et al., 2006). However, the mechanism by which cells escape checkpoint has not yet been elucidated.

In response to DNA damage and DNA replication stress, cells activate surveillance pathways called cell cycle checkpoints. Activation of these checkpoints slows or arrests cell cycle progression to ensure appropriate time for DNA repair. These biochemical networks contain a class of protein, named mediators or adaptors, which promote functional interaction between sensor and effector proteins. Claspin is one of these mediators. In *Xenopus* and human cells, Claspin is phosphorylated and associates with Chk1 in response to DNA damage and replication stress. Recently, it has been shown that following aphidicolin (APH) treatment allows cells to escape from the checkpoint arrest, so that mitosis occurs despite the presence of unreplicated DNA in *Xenopus* egg extracts.

In this study, we provide evidence that *Claspin* inhibition by RNA interference lead to chromosome aberrations and fragile sites expression. Following prolonged APH treatment, Claspin synthesis decrease, as a consequence, cells complete cell cycle despite the ongoing presence of damaged DNA. These data strongly support the notion that normal cell cycle checkpoints activity and DNA repair systems are linked through complex pathways of gene expression and that their alteration predisposes to genomic instability and contributes to cancer.

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Paroxysmal Nocturnal Hemoglobinuria

Rosario Notaro

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Paroxysmal nocturnal hemoglobinuria (PNH) is a rare acquired blood disorder characterized by intravascular hemolysis, tendency to thrombosis, and variable degrees of bone marrow failure. PNH is due to the clonal proliferation of a hematopoietic stem cell (HSC) in which a somatic mutation has inactivated the X-linked gene *PIG-A*. The resulting deficiency of glycosylphosphatidylinositol (GPI)-linked proteins on the surface of the progeny of the mutated HSC explains hemolysis and thrombophilia: but it does not explain the bone marrow failure and the expansion of PNH (GPI-negative) clone. Clinical observations, in vitro studies, and data from PNH mouse models indicate that GPI-negative HSC do not have an absolute growth advantage. In addition, very small GPI-negative clones exist in healthy subjects, but only in PNH patients GPI-negative cells expand and contribute substantially to hematopoiesis. A plausible model that could explain both bone marrow failure and the PNH clone expansion is that normal (GPI-positive) HSC are the target of a selective auto-immune attack to which PNH HSC are resistant. Auto-reactive T cells against HSC are responsible for idiopathic aplastic anemia (IAA); the close relationship of PNH with IAA has suggested that they may be present also in PNH. Indeed, the analysis of the TCR repertoire has revealed an increased frequency of expanded T-cell clones in PNH, similar to that observed in IAA. The identity of the putative auto-reactive T cells and of their targets remains unknown. However, we have found that in PNH patients the expansion of CD8+CD57+ T cells is relatively common. In addition, by a systematic sequence analysis of the TCR-beta molecules we have shown that in PNH patients CD8+CD57+ T cells are oligoclonal and that more than two-thirds of patients share, on this T-cell population, a set of highly homologous TCR-beta molecules (clonotypes). These findings are consistent with the presence in PNH patients of an immune process driven by the same (or similar) antigen(s): probably a non-peptide antigen, because patients sharing clonotypes do not all share identical HLA alleles. These findings provide strong support to our hypothesis that the expansion of the GPI-negative blood-cell population in PNH is due to selective damage to normal hematopoiesis, mediated by an autoimmune attack of CD8+CD57+ T cells against a non-peptide antigen(s) that could be the GPI anchor itself. In order to test directly the hypothesis that the GPI anchor itself is the auto-antigen targeted in PNH, it is crucial the availability of the human GPI anchor. Mammalian GPI has never been synthesized; however, we have been able to design a convergent and versatile synthesis of the core structure of mammalian GPI relying on a reduced number of protective group manipulations. The oligosaccharidic portion was assembled employing diastereo selective glycosylation reactions to achieve, under very mild conditions, the desired alpha glycoside linkages. The reduction of the azido- to amino group and the final removal of benzyl protecting groups revealed to be critical and time-demanding steps. After the optimization of the analytical methods, the correct molecular structure of the synthesized GPI has been fully confirmed.

The availability of the GPI molecules provides us a powerful tool to directly investigate the peculiar autoimmune pathogenesis of the clonal expansion observed in PNH. In addition, it is possible that similar autoimmune mechanisms could be responsible of the clonal expansion in others acquired clonal cytopenias related to IAA and PNH, such as myelodysplastic syndromes (MDS) that are much more frequent than PNH and far more prone to evolve to acute leukemia.

Compliance with standards of care. The experience of the Istituto Toscano Tumori (ITT)

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Evidence-based guidelines are increasingly regarded as essential in cancer care and indicators are measures that can be used to monitor all aspects of the health care process or outcome, including compliance with guidelines. The first aim of the Istituto Toscano Tumori was to produce shared guidelines for the management of the more frequent types of solid tumors; as well as indicators of the standards of cancer care. A major challenge was that of collecting information on the entire population of Tuscany: this involved tracing all cancer patients who are resident in the Region, whether they were cared for by public or private providers. For this purpose the cancer registry as essential epidemiological tool.

Methods:

The study included all the resident population of the Tuscany Region, which had in 2004 a population of 3.585.000 inhabitants. In total 3016 breast, 2617 lung and 3304 colorectal cancer incident cases were considered. For the 2006 year, breast, colorectal and lung cancer patients were selected as reported by the hospital discharge notes. Results were characterized at three sub regional areas, but also made available to each local health authority .

Quality indicators were identified through ITT recommendations and discussed with a group of specialists of the regional cancer network.

Each indicator was correlated to the clinical recommendations and in some cases, particularly for breast cancer, a benchmark was suggested. The quality score was calculated from Registry data in 2004 and compared with that calculated from medical charts data in 2006. . Data were analyzed for the whole region, macro area and local health authority for patients younger than 75 years and those 75 year old and older.

Results:

In total, there was a consensus about 32 indicators: 10 for lung cancer, 12 for breast cancer, 6 for colon and rectum, 4 for rectal cancer only, the cancer site presented in this communication. Size, nodal status and grading were retrieved in the greatest number of cases and allowed for an evaluation of treatment by level of gravity at presentation. Main average results of the Tuscany Region are presented in Tables 1 a,b,c

Discussion:

The main result of this study is that a population-based evaluation of the quality of cancer care using cancer registry population-based data and major computerized information systems is feasible, also in large areas. The use of the 2004 cancer registry data allowed for the estimate for the whole target population and the survey 2006, also population based, has confirmed the validity of the main result supporting the possibility of an improvement in some indicators, especially those related to the diffusion of new technologic approaches. The development of e-medicine is not homogeneous in the Tuscany Region and in order to be able to report for the whole regional system we need to rely on the universal , basic information, especially pathology records, which is today available . Of course there is a mix of providers

which are available to the citizen resident in a local authority area (local versus the university hospital in the macroarea, for example) but the main responsibility of the local health authority is to provide a good standard of quality in every setting of care the subject can choose. The analysis by provider will in the future offer more insight about performance of each player of the curing team..

Population based cancer registries provide a large numbers of cases and include all persons in the community; they provide the date at incidence and the living status of the subject , essential information for the evaluation of the clinical pathway of each cancer case. The result in the Tuscany regional system confirmed a good homogeneity between areas and globally the estimate of the selected indicators showed a good performance. The estimates for 2004 and 2006, which were based on a sample, were comparable and the performance was increasing for indicators evaluating the diffusion new technological approaches , like sentinel lymph node and biomarkers. It is interesting to note the different attitude towards preoperative diagnosis in some areas of the Tuscany Region. This difference persisted in 2006, showing that there is professional cultural perspective which determines the divide in medical procedures. This difference was the object of discussion between clinicians when reported in a project final meeting.

Further work is needed to develop specific quality measures about structural data as hospital volume or access to care; moreover, indicators will need to be continually updated and revised with the improvement of scientific knowledge and information system.

Intelligent radiation

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Intelligent systems are intended as “able to learn from experience to reach an appropriate decision or react in an appropriate manner to a set of specific stimuli. ... Such an ability to change, adjust, and modify their behavior to suit a new set of conditions or objectives is characteristic of adaptive systems. This means that intelligent systems are often adaptive too” [1]. Even the most sophisticated radiation treatments presently in use are not “intelligent”, according to this definition. However, radiation therapy of human diseases, mainly cancer, became more and more adaptive during the last 30 years, according to the requirement of selectively hitting tumor volume with high radiation doses and sparing healthy tissues. This evolution started with the availability of CT scans, and the first algorithm specifically designed for CT pixel-based calculation, using 3-D information, was published in 1978 [2]. Three-Dimensional “Conformal” Radiation Therapy (3D-CRT), that is, adaptive to the shape of the target, is the present standard of radiation treatment, thanks to the evolution in linear accelerator and computer planning technology. The subsequent evolutionary step occurred in the late eighties of the last century [3], allowing the possibility to deliver both uniform doses to concave volumes, and simultaneously boosting regions requiring a higher dosage, through the so-called Intensity-Modulated Radiation Therapy (IMRT).

Nowadays many sophisticated algorithms are available, allowing very fast and accurate dose calculations even in case of very complex techniques. Last developments in IMRT include helical TomoTherapy, in which patient is irradiated following the same principle of helical CT, and fast delivery intensity modulated arc therapy (Rapid Arc, VMAT), in which patient is irradiated while dynamically changing gantry angle, field shape and dose rate; both will be available at ITT within the next months. Using these advanced techniques it is possible to optimize the irradiation taking into account radiobiological functions such as the Equivalent Uniform Dose (EUD), the estimated Tumor-Control Probability (TCP) and Normal Tissue Complication Probability (NTCP), and also to include diagnostic data coming from functional imaging, thereby taking into account bio-molecular information.

On board imaging systems integrated with the linear accelerator (cone beam CT) or position verification systems like stereo X-rays units plays a fundamental role in modern radiotherapy. Through these three-dimensional imaging systems it is possible to track the target shape and position, both between different fractions and during the course of a single fraction; the irradiation technique and the patient position may be corrected taking into account these information (Image-Guided Radiation Therapy, IGRT). Because these three-dimensional imaging systems make it possible to follow space modifications of the target over time due to inherent motion, e.g. to breath or to the filling state of hollow organs, irradiation might, at least in theory, follow the tumor in real-time, for example through a robotic-arm mounted accelerator and a frameless system of coordinates. In other words, irradiation may be adapted in real time to tumor shape and position (ART, Adaptive Radiation Therapy). Presently, in fact, the definition of ART is restricted to approaches that transform a static process of irradiation into a dynamic one.

Inside ITT many experiences of advanced radiotherapy are ongoing. Intensity Modulated Radiation Therapy, together with cone beam CT, is extensively used for head and neck and prostate treatments, allowing for dose escalation protocols respectively up to 70 Gy and 80 Gy with limited unwanted side effects. A phase III randomized protocol of Accelerated Partial Breast Irradiation with IMRT is ongoing in Florence Radiotherapy Department, and other ITT centers from will join this protocol in the next future. Pleural mesothelioma is another disease treated with IMRT at ITT; only few centers in Italy treat this disease, due to the complexity of the technique and the high risk of severe side effects. Experiences with advanced radiotherapy within ITT are briefly illustrated.

Whether these breakthroughs might improve survival results of cancer therapy, is not yet demonstrated in terms of evidence-based medicine [4], but a favorable impact is soundly assessed at least as far as concerns toxic effects of irradiation thus allowing, for instance, safe hypo-fractionated treatments delivered during a rapid course.

All the above technology is based on “hard-computing”, that is, purely numeric, integral-differential approaches. Whether different systems (“soft-computing”) based on “artificial intelligence” (e.g.: non-linear function approximations using Artificial Neural Networks, algorithms based on Fuzzy Logic, etc.) might effectively contribute to the progress of radiation therapy, it is still the subject of some very interesting, but mainly theoretical contributions [5].

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Membrane Gamma-glutamyltransferase: at the crossroads of tumor progression, drug resistance and drug targeting

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Gamma-glutamyltransferase (GGT) is often significantly increased in human malignancies. Previous studies have repeatedly documented a role of GGT in cellular redox balance, through the production of a low but persistent oxidative stress (1, 2). When GGT-overexpressing cells were incubated in the presence of GGT substrates and a source of catalytic iron, increased levels of DNA damage were observed (Comet assay). This phenomenon was suppressed by specific GGT inhibitors such as ABBA, as well as by iron chelator DFO or antioxidants BHT and Trolox C. Interestingly, higher levels of basal DNA damage were observed in GGT-overexpressing cells as compared to low-expressing ones. These results suggest thus a role of GGT expression *per se* in tumor progression (Corti, Pompella et al. 2009. manuscript submitted).

On the other hand, GGT has been implicated in cancer drug resistance, as it participates in the reconstitution of cellular antioxidant/antitoxic defences (3). However, the enzyme can play as a factor both in drug resistance and drug sensitivity, as documented by the results of our latest studies:

i) the protective effects of GGT against cisplatin cytotoxicity are independent of intracellular glutathione, and depend rather on extra-cellular reactions of cisplatin with GGT-derived thiol metabolites, leading to formation of adducts which are far less cell-permeable (4);

ii) on the contrary, in the case of 4-[N-(S-glutathionyl-acetyl)amino] phenylarsinous acid (GSAO) – a promising anti-angiogenic drug – cell membrane GGT activity acts as a sensitizing factor. The gamma-glutamyl residue of GSAO in fact is cleaved at the surface of GGT expressing cells, thus producing the metabolite GCAO. This information can also explain GSAO kidney toxicity at high doses: GGT is in fact expressed at high levels in tubular epithelia (5);

iii) recent observations highlight NO and NO-donor agents (e.g. S-nitroso-glutathione, GSNO) as “chemosensitizing agents”, capable of potentiating the action of several anticancer drugs (Sullivan et al., *Curr Pharm Des.* 2008; 14: 1113). As GGT possesses the selective ability to metabolize GSNO – thus releasing its NO load – its expression may well be exploited to target NO to GGT-expressing tumor cells. By investigating the kinetics of the reaction we have found a K_m of GGT for GSNO of approx. 0.4 mM, comparable with the K_m value for glutathione (Angeli, Pompella et al., *Arch. Biochem. Biophys.* 2009; 481: 191), which confirms the feasibility of using GSNO as an efficient pro-drug in order to perform selective “NO treatment” of GGT-expressing tumors. Future studies will substantiate the usefulness and applicability of such approach to therapy.

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Targeting mitochondria in glial tumors

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High grade gliomas are one of the most difficult cancers to treat and despite surgery, radiotherapy and temozolomide-based chemotherapy, the prognosis of glioma patients is poor. Resistance to temozolomide and diffuse growth are the major barrier to effective therapy. Alternative therapeutic approaches have been shown to be ineffective for the treatment of genetically unselected glioma patients. Thus, novel therapies are needed.

Mitochondria are emerging as a promising target to combat cancer with regard to their role in regulating cell death/survival processes as well as for their involvement in cellular metabolism processes that are frequently altered in cancer cells respect to normal counterparts.

In our laboratory we have undertaken researches aimed to: (a) pharmacologically target the mitochondrial permeability transition pore to produce cancer cell death [1-3]; (b) genetically manipulate mitochondrial proteins to study their role in tumor aggressiveness and thus to identify putative gene therapy target to limit tumor growth and diffusion [4,5].

Among the mitochondrial proteins to be studied as potential therapeutic targets, the adenine nucleotide translocase (ANT) family is particularly interesting due to their role in mediating cytoplasm/mitochondrial matrix cross talk in relation to different cellular functions including ATP/ADP co-transport, basal proton conductance, mitochondrial permeability transition and heme biosynthesis.

In this context we evaluated the ability of some agents, that on the basis of indirect literature data were reported to target ANT, to act as cytotoxic drugs in a temozolomide resistant human glioblastoma cell line (ADF cells). We demonstrated that these agents reduces tumor cell viability, by inducing mitochondrial permeability transition through the opening of the mitochondrial permeability transition pore and promoting cancer cell apoptosis and autophagy.

Moreover, we have recently undertaken a study on ANT isoform expression and role in human glioblastoma. ADF cells exclusively express ANT1 and 2 isoforms and thus we concentrated our functional studies on these proteins. We demonstrated that the downregulation of isoform 1, by short interfering RNA (siRNA), strongly reduced cell viability by inducing cell apoptosis. ANT2 neither increased nor reduced ANT1 depletion-dependent effect on cell viability. ANT1 siRNA-induced apoptosis was not due to the impairment of ATP/ADP co-transport function since we demonstrated that ADF cells exclusively depend on glycolysis and that the pharmacological-induced arrest of the co-transport function did not affect cell survival and growth. We can hypothesize that ANT1 silencing impairs the basal proton conductance producing mitochondria hyperpolarization which, in turns, triggers ROS production. In fact, we demonstrated that, as a consequence of ANT1 siRNA, ADF cells show:

- i) high lysosome instability, probably due to lipid membrane peroxidation;
- ii) an increase in glucose consumption, indicative of an attempt to increase the detoxification activities;
- iii) a reduced tolerance to exogenous pro-radicals, indicative of a saturation of the endogenous detoxifying system.

These preliminary results are particularly promising taking into account that the ANT isoform expression is generally profoundly modified in tumor cells with respect to normal counterparts. Moreover, the effects produced by the downregulation of a specific ANT isoform may differentially affect cancer cells and normal counterparts due to their completely different metabolic state. For these reasons, future aims will be to (i) analyze the effect of ANT1 downregulation in other glioma cell lines to verify that its silencing may affect the growth of different brain tumors and (ii) to study ANT isoform expression and ANT1 downregulation effects in primary culture of normal human astrocyte to prove a tumor-cell specific effect.

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Hedgehog-Gli signaling controls neural stem cell and tumor cell numbers

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It is not known how strict controls of cell numbers are achieved during development and how they are deregulated in cancer. The Hedgehog-Gli (Hh-Gli) signaling pathway plays important roles in development, homeostasis and cancer. During early brain development Hh-Gli modulates the proliferation of neural precursors in the dorsal brain, while later it regulates the behavior of neural stem cells in neurogenic niches. It has been also involved in the genesis of tumors, including those of the brain, and in controlling self-renewal of brain cancer stem cells. However, it remains unclear if the zinc finger transcription factor Gli1, the final mediator of Hh signaling, controls stem cell number and how its activity is normally restricted. We show that GLI1 expression increases stem cell numbers *in vivo* and *in vitro*. In contrast, p53, the major tumor suppressor, inhibits GLI1-driven neural stem cell self-renewal, proliferation and tumor growth. p53 inhibits GLI1 and, in turn, GLI1 represses p53, establishing a negative regulatory loop that is central to control neural stem cell numbers and to prevent GLI1-driven tumorigenesis.

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Molecular mechanisms of HCV-related Lymphoma genesis

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Hepatitis C virus (HCV) has been recognized as one of the human oncogenic viruses. The double hepatic and lymphatic tropism of the virus is reputed to lead to both hepatic (hepatocellular carcinoma, HCC) and/or lymphatic (non-Hodgkin's lymphoma, NHL) malignancies. The mechanisms underlying viral oncogenesis, -especially HCV-related lymphoma genesis - are largely unknown. The causal role of HCV in some B-cell NHL has been proved by epidemiological studies showing a significantly increased risk for NHL in HCV-positive patients, as well as by the regression of NHL after successful antiviral therapy. Our research group had been investigated the molecular mechanisms of HCV-related lymphoma genesis for over a decade, starting from early nineties with the first evidence of HCV infection and replication in lymphatic cells¹. The analysis of the pathogenetic consequences of the viral lymphotropism has been a major topic of our research group and led to the definition of a strong association between the presence of HCV RNA in mononuclear cells and the presence of a pre-neoplastic, B-cell lymphoproliferative disorder known as mixed cryoglobulinemia (MC). HCV-related MC was an unique model to define the possible mechanisms involved in the evolution towards the frank neoplastic disease. In particular, (14;18) translocation was shown to be associated with HCV-related lymphoproliferative disorders (LPDs)². This chromosomal rearrangement involves the proto oncogene *bcl-2* and leads to the over expression of this survival factor. These data provided evidence for the abnormal survival of pathogenetic B-cell clones underlying MC and supporting the hypothesis of a key role played by the inhibition of B-cell apoptosis in HCV-related lymphoma genesis. More recently, we characterized an interesting model represented by the occult infection of lymphatic cells in HCV-MC subjects who cleared the virus from serum and liver after an (apparently) successful antiviral therapy³. The persistence of the virus in the sole lymphatic compartment was strictly associated with the persistence of clinical manifestations of this LPD. Furthermore, the presence and maintaining of t(14;18)-positive clones was significantly more frequent in those patients who carried HCV in the PBMC. These results suggest a direct relationship between the viral lymphotropism and the pathogenesis of such lymphoproliferative disease and also suggested more appropriate clinic-therapeutic approaches to HCV positive patients with LPDs⁴.

On this basis, as planned in the research project, we further developed the model of the isolate lymphatic infection increasing the analysis of patients who achieved a sustained virological response after antiviral therapy. We studied 156 patients and we found an occult infection in 20 cases, mostly with MC or other B-cell LPDs. Interestingly, the possibility of a reappearance of an overt HCV infection after several years of complete absence of HCV viraemia was shown, suggesting that occult PBMC infection could be considered a risk factor for viral reactivation as well as a trigger to maintain the LPD.

Interesting and promising preliminary results were obtained by the analysis of the role of B-cell specific factors in the pathogenesis of HCV-related LPDs. Two members of the TNF-super family, named BAFF and APRIL, have been involved in the pathogenesis of several LPDs including some types of B-cell NHL. A significantly higher serum concentration of BAFF, but not of the analogous APRIL, was found in the serum of patients with HCV-related LPDs, mainly in patients with NHL, when compared to pathological and healthy controls⁵.

This phenomenon was partly due to the elevated transcription of the BAFF mRNA in the monocyte/macrophage compartment of these patients. A contribution to the elevated expression of the BAFF gene was also attributed to host genetic factors. In fact, it has been shown that polymorphic variants of the BAFF promoter sequence are able to induce an enhanced transcription of the gene; the presence of this mutated form of BAFF promoter was significantly more frequent in patients with HCV related LPDs, suggesting a mechanism for the expansion of B-cell clones in these subjects.

Finally, we analyzed the possible synergic action of the combined infection by HCV and HBV in the pathogenesis of virus-related neoplasia. Preliminary results obtained in 12 patients with HCV-related NHL showed the possibility of a HBV occult coinfection strongly suggesting the interest of such a working hypothesis.

In conclusion, during the first semester of the research project, according to the scheduled research plan, the experimental work produced some preliminary results that encourage us in continuing the investigation and the efforts to define some aspects of the multifaceted lymphomagenic process and the potential pathogenetic role of HCV infection.

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