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LINE-1-encoded reverse Transcriptase as a target in cancer therapy

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1. ABSTRACT

LINE-1 elements account for about 17% of the human genome and harbour two open reading frames: ORF1, encoding a 40 kDa RNA-binding protein, and ORF2, coding for a 150 kDa protein with reverse transcriptase (RT) activity. LINE-1s are highly expressed in embryos and tumor cells while being virtually silent in differentiated tissues and, consistently, both ORF-1p and ORF-2p have been detected in human cancers. RT-encoding ORF2 is expressed early in pre-neoplastic lesions suggesting that RT expression may be a potential cause, rather than a consequence, of cancer onset. Experimental data emerging from *in vitro* and *in vivo* studies confirm this view. Preclinical work showed that RT inhibition reduces proliferation, promotes differentiation of cancer cells and antagonizes tumor progression in murine models. Moreover, a recent phase II trial on metastatic hormone-resistant prostate cancer patients has confirmed the anticancer efficacy of RT inhibitors. Together, these data indicate that LINE-1-encoded RT emerges as a potential therapeutic target for a large spectrum of cancers and RT inhibitors as effective tools in a novel anti-cancer, non–cytotoxic, differentiation therapy.

2. INTRODUCTION

2.1 The retroelement landscape of the human genome

In recent years the advent of high-throughput technologies has produced a flood of information that has shifted the focus of biological research from a gene-centric to a genome-wide view, and has disclosed novel and unexpectedly complex genomic landscapes. A major finding was that the protein-coding sequences are limited to a mere 1.2.% of the human genome, while the vast majority is constituted by non-coding sequences (1), grossly indicated as "dark matter".

A large proportion of the non-coding genome comprises various families of retrotransposons, which are a major source of structural and functional genomic variations and are increasingly recognized as regulators of genomic functions.

Retrotransposons are mobile genomic elements that retrotranspose throughout the eukaryotic genomes via reverse transcription of RNA intermediates. A reverse transcriptase (RT) activity fuels retroelement mobilty and concomitantly promotes their copy number amplification. The human genome contains four major families of retrotransposons, i.e. LINE-1, HERV, Alu and SVA, which collectively account for about 45% of the genome (1). LINE-1 and HERV encode the RT that is required for their own retrotranscription, and hence are autonomously retrotransposing elements. In contrast, Alu and SVA lack the RT coding genes and exploit the LINE-1 mobilizing machinery to retrotranspose (2).

LINE-1 (fig1) is the largest retrotransposon family, accounting for about 500.0.00 copies, corresponding to 17% of the human genome. Each copy encodes a bicistronic RNA transcript that

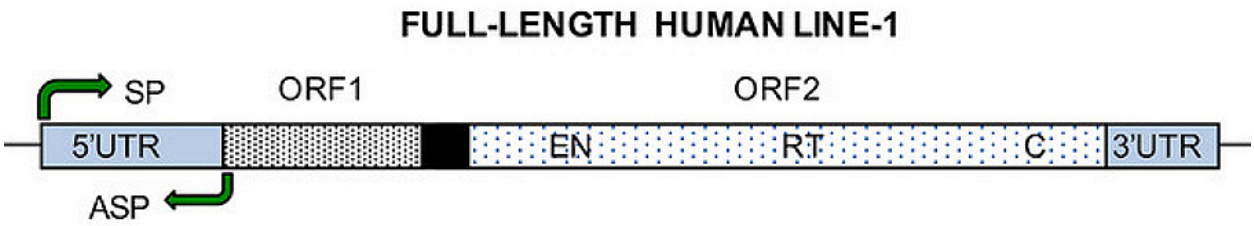

Figure 1. Structure of the human LINE-1 retroelement. 5'-UTR and 3'-UTR, untranslated regions; ORF1 and ORF2, open reading frames 1 and 2; the polycitronic ORF2 encompasses regions encoding EN, endonuclease (EN), reverse transcriptase (RT) and cystein-rich (C) domains. The black box represents the intergenic spacer between the two ORFs. SP, sense promoter; ASP, anti-sense promoter.

is translated into a 40 kDa RNA-binding protein (ORF-1p) and a 150 kDa protein (ORF-2p), the latter with endonuclease (EN) and reverse transcriptase (RT) activities. The vast majority of LINE-1 elements are 5'-truncated and unable to retrotranspose, but nevertheless transcriptionally active (reviewed in 3). Only a subpopulation of 80-100 LINE-1 copies are full-length and retrotranspositionally competent (4).

LINE-1 retrotranspositions are major cause of genome reshuffling (5) with potential mutagenic effects. Growing evidence indeed implicate LINE-1 insertions in an ample spectrum of cellular abnormalities and pathologies, including tumors (6). Consistently with this, massive new insertions have been identified and mapped in the genomes of several human cancers, e.g. lung (7), colorectal (8,9), oesophagus (10), pancreas (11), gastric (12) and ovary (8,13) carcinoma, glioblastoma, multiple myeloma (8), and hepatoma (14). However, the question remains unanswered as to whether these bursts of LINE-1 retrotranspositions accompany or cause tumorigenesis or, in other words, whether LINE-1 insertions are "driver" mutations with potentially causative roles in tumorigenesis or only irrelevant "passenger" mutations (15).

A second important discovery with relevant implications indicated that the genome dark matter is pervasively transcribed in non-coding RNAs (16), constituted by a heterogeneous population of various small and long non-coding RNA families (17,18). A wealth of data now reveals that non-coding RNAs are key players in genome-wide regulatory epigenetic networks involved in countless cellular functions (reviewed by 17, 19, 20).

LINE-1 transcription undergoes a peculiar bimodal regulation: it is virtually silent, or expressed at very low basal levels, in healthy differentiated somatic cells and tissues, while being highly up-regulated in embryonic (21-23) and cancer cells and tissues (reviewed in 24), concomitant with an overall genomic hypomethylation (25). The only exception are retrotransposition-permissive neurons, in which LINE-1 activity emerges as a constitutively activated function (reviewed in 26). With this significant exception, available data show that LINE-1 expression is typically active in cellular contexts characterized by low differentiation and high proliferation rates, identifying it as a hallmark of human cancer, and suggesting its possible involvement in tumorigenesis.

3. LINE-1-ENCODED RT CHARACTERIZES CANCER ONSET AND PROGRESSION

A considerable amount of data consistently shows that LINE-1-encoded proteins, i.e. ORF1p and ORF2p, are overexpressed in cancer.

Immunohistochemical (IHC) studies depicted ORF1p overexpression in colon, renal, hepatocellular, lung, breast, pancreatic, biliary tract carcinoma, lymphoma and pediatric malignant germ cell tumours (27,28). An anti-ORF2p polyclonal antibody detected ORF2p-encoded EN in gastric cancers and their lymph node metastases (29), and in breast cancer (30). Interestingly, the nuclear localization of either ORF1p alone (31), or of ORF1p and ORF2p (30), was associated with poor prognostic outcome breast cancer. The early expression and activity of RT protein, and their increase in amount and nuclear localization were further documented using a Py-MMTV transgenic mouse model of breast cancer progression (32). Consistent with the IHC detection of LINE-1 ORF2p overexpression, the RT enzymatic activity encoded by ORF2p has also been detected in RNA-based *in vitro* assays in a variety of carcinoma, sarcoma and leukemia cell lines (33,34) but not in normal cells. The recent development of a highly efficient monoclonal antibody has further improved the detection of ORF2p in human bioptic samples (35). That has revealed that ORF2p is highly expressed in colon, prostate, lung and breast carcinoma bioptic tissues but not in their healthy counterpart tissues. Most interestingly, both colon and prostate carcinoma show elevated ORF2p expression in very early stages, before the appearance of typical histological features of carcinoma lesions, and even in pre-neoplastic lesions, e.g. prostate intraepithelial neoplasia (PIN) and transitional colonic mucosa (fig2), in good correlation with the early occurrence of massive genomic hypomethylation and increased retrotransposon expression in both colonic mucosa

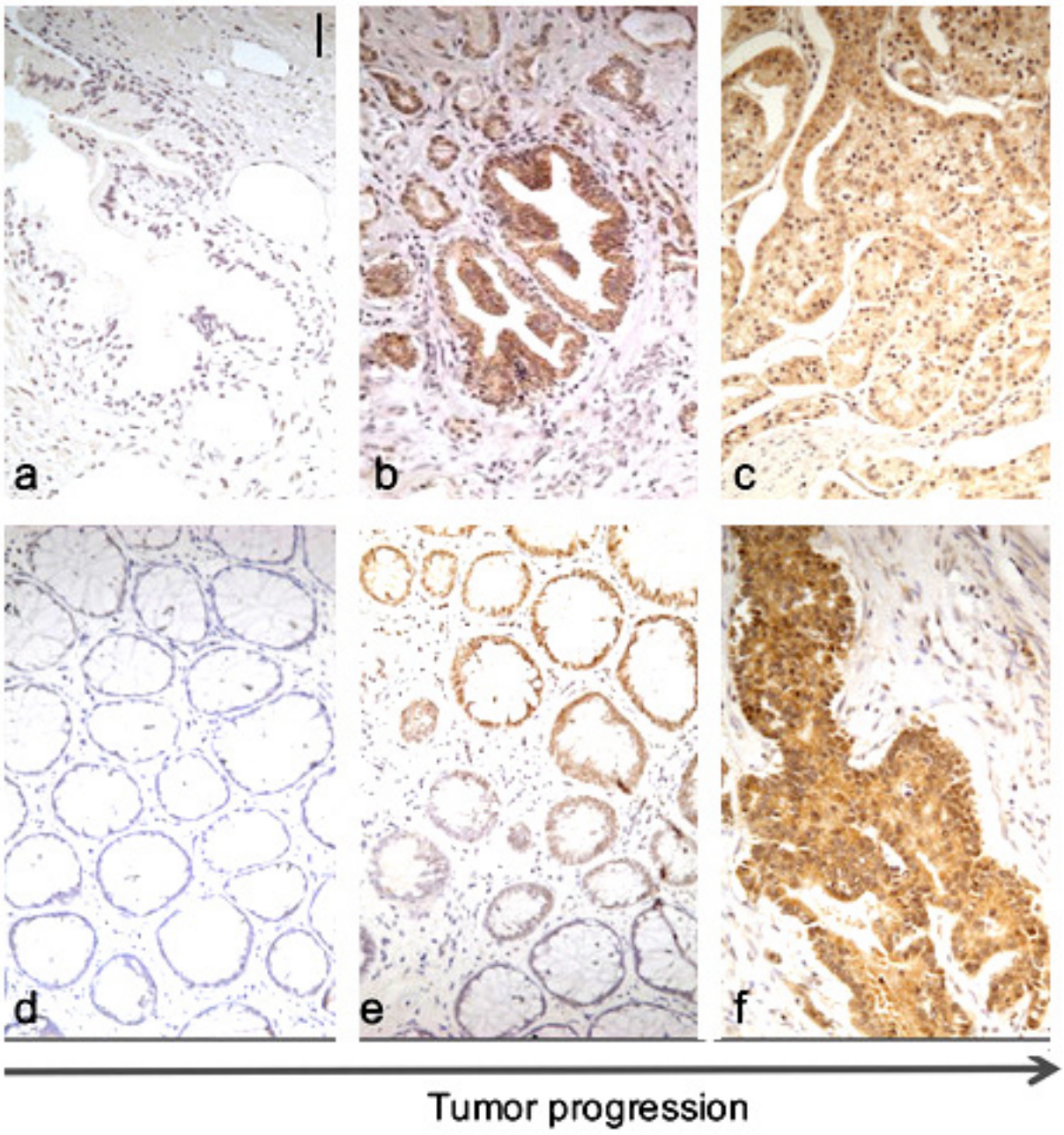

Figure 2. Immunohistochemical staining of ORF2p in prostate and colon cancer stages. Representative sections from: a) normal prostate; b) prostatic intraepithelial neoplasia (PIN); c) prostate adenocarcinoma with Gleason pattern 4; d) normal colonic mucosa; e) transitional mucosa and f) colon adenocarcinoma. ORF2p signal intensities (in arbitrary units) were highly significantly different in transitional compared to normal mucosa (see 35).

and PIN (36,37). The early expression in pre-neoplastic tissues rules out the possibility that RT-encoding ORF2p is expressed in consequence of tumor transformation, rather suggesting an active role in tumorigenesis.

3.1. RT inhibition as a novel non-cytotoxic anti cancer therapy

Indications favouring a causative role of LINE-1-encoded RT in tumorigenesis emerged from the finding that RT inhibitors counteract the growth of cancer cells and antagonize cancer progression in animal models. Our group first discovered that nevirapine and efavirenz, two nonnucleoside RT inhibitors (NNRTis) targeting the HIV-1-encoded RT, currently used in AIDS therapy, reduces cell proliferation and promotes differentiation of a variety of cancer cell lines of unrelated histological orgin (carcinomas, sarcomas) (33,38,34). Those original findings were independently confirmed and extended to a larger spectrum of cancer cell lines (39-43). Moreover, similar effects were obtained in cancer cells treated with nucleotide inhibitors (NRTis), i.e. abacavir (44), azidothymidine and didanosine (45).

In contrast, no significant effects were observed when treating non-expressing RT normal cells (46,43). In general, RT-inhibited cells retained a low proliferating, redifferentiated phenotype as long as they were exposed to the inhibitors, but quickly reverted to their highly proliferating, dedifferentiated condition upon discontinuation of the treatment. These data indicate therefore that "differentiation" is not a stably acquired condition upon RT inhibition, but rather a reversible trait (38). RNA interference (RNAi)-mediated down-regulation of LINE-1 expression generated identical effects to those observed with RT inhibitory drugs in human melanoma cells (38,47): the finding that post-transcriptional silencing of LINE-1 elements "phenocopies" the inhibition of their product rules out the possibility that low proliferation and morphological differentiation are off-target effects of the pharmacological inhibitor. Based on these data, the functional knock-down of LINE-1 expression, alone, is sufficient to counteract the cell tumorigenic potential and creates favourable conditions that revert cells from a tumor to a "normal" state.

The anti-cancer efficacy of efavirenz was further confirmed in *in vivo* assays using murine models inoculated with various human cancer cell lines (38,47). Daily treatment of animals with efavirenz antagonized, or significantly delayed, the progression of tumors, which was resumed upon the discontinuation of the treatment. This reversibility in animal models confirmed the data from cultured cancer cell lines. Consistent with this, permanent RNAi-mediated down-regulation of LINE-1 drastically reduced, or abolished, the tumorigenic potential of melanoma cells in nude mice (47). On the whole, inhibition of LINE-1-encoded RT results in effective reduction of tumor invasiveness, represses cancer progression and promotes differentiation, provided that the cancer cells, or cancer animal models, are continuously exposed to the drug.

Human case reports largely confirmed the preclinical data showing: i) the anticancer potential of nevirapine in HIV-negative patients with thyroid cancer (48,49), ii) an extended long-term survival of an HIV-infected patient with small cell lung cancer treated with NNRTI-based HAART (highly active antiretroviral therapy) (50), and iii) the regression of lymphomas (51,52).

Recently, a phase II human trial using efavirenz on a cohort of metastatic patients with prostate primary carcinoma showed non-progression (assessed by PSA levels and bone metastases) when efavirenz reached an optimal blood concentration (53). Together, therefore, preclinical and clinical data provide evidence that RT inhibition is a potentially effective tool in a novel anti-cancer therapy against a large spectrum of human cancers with non-cytotoxic effects on non-cancer cells.

3.2. An RT-dependent tumor-promoting mechanism is active in cancer cells

Based on the data reported above, LINE-1 retrotransposons may operate at two possible levels in tumorigenesis: either as a source of potentially mutagenic genomic insertions, or via induction of RT-mediated epigenetic variations. The findings that LINE-1-derived, RT-containing ORF2: i) is expressed early in pre-neoplastic stages, ii) shows increased expression throughout cancer progression, and, most significantly, iii) is required to maintain the cancer state, as RT inhibition antagonizes cancer progression and reverts cancer cells to a "normal "state, converge to support the view that LINE-1 RT plays a causative role in the genesis and progression of cancer. These results also minimize - if not exclude - a role for retrotransposition-dependent mutagenic insertions in the onset and progression of cancer, which emerges instead as an eminently epigenetic process. This conclusion provides further support to the long-lasting notion that "epigenetics wins over genetics" in cancer (54). The finding that RT inhibition causes a substantial reprogramming of the transcriptional landscape in cancer cells, involving both protein coding and non-coding RNAs (46), is consistent with this idea. Indeed, RT inhibition preferentially affects the miRNA profile ("miRNome"), with a profound impact on the cellular global transcriptome; in particular, efavirenz administration to cancer cells restores the normal profile of a sub-group of miRNAs, classified as metastamiRs, with key roles in tumor progression and metastasis. Similarly, LINE-1 silencing in breast cancer cells is reported to alter the expression of many miRNA species (in particular, members of the let-7 family), and of some piRNAs that can potentially regulate gene expression (55).

Building on these lines of evidence, we have proposed a model whereby LINE-1-encoded RT modulates the biogenesis of miRNAs. Experimentally, RT inhibitors alter the balance between the production of regulatory double-stranded RNAs (dsRNAs) and RNA:DNA hybrid molecules (46,56). Based on these observations, the model predicts that the highly abundant RT activity expressed in cancer cells inhibits the formation of ds RNAs, including miRNA precursors, by actively reverse-transcribing the RNA complementary strands, yielding the preferential formation of RNA:DNA hybrids. This process ultimately impairs the normal biogenesis of miRNAs with a direct impact on global gene expression. The model is consistent with previous observations that LINE-1-derived miRNAs (57) and siRNAs (58) are globally down-regulated in cancer compared with normal cells. RT inhibition prevents the formation of the hybrid molecules, re-establishes a non-pathological profile of regulatory RNAs, thus normalizing the cellular transcription landscape, and hence restores the differentiated phenotype of cells.

To summarise, a LINE-1 RT-dependent tumor promoting mechanism:

1. is a constitutive feature of many cancer types, regardless their histological origins,
2. is repressed under permanent LINE-1 silencing or continuous exposure of cancer cells to anti-RT drugs, indicating the anti-cancer efficacy of RT inhibitors
3. is resumed when the RT inhibitory treatment is discontinued.

On the whole, these data suggest that RT may be regarded as a "universal" therapeutic target in an ample spectrum of human neoplasia.

4. THE EMBRYO CONNECTION

The ubiquitous presence of the RT-dependent mechanism in cancer raises the puzzling question of its origin in human cells. Our parallel studies to investigate this aspect in depth showed that the LINE-1-encoded RT activity: i) is present in mammalian spermatozoa (59), ii) is physiologically activated in both zygotic pronuclei, iii) is up-regulated in early preimplantation embryos, concomitant with the expansion of LINE-1 copy number, and iv) is progressively abrogated in later developmental stages (22). As in cancer cells (see above), the pharmacological inhibition of RT activity (60), or LINE-1 post-transcriptional silencing (61), both caused the arrest of embryo development at the 2-4-cell stages. These data reveal that an RT-dependent mechanism physiologically operates in early embryogenesis, is strictly required for development, and is absent from adult healthy cells and tissues. This suggests that the RT-dependent tumor-promoting mechanism represents the unscheduled reactivation of the RT-dependent embryonic mechanism in adult cells (reviewed in 62). RT reactivation causes the functional resurrection of genetic networks - typically expressed in early embryos - which generate aberrant transcription profiles in adult cells, ultimately promoting their transformation. Under this light, cancer would not arise in consequence of incremental genomic mutations, but rather as the unscheduled resurrection of an ancestral RT-mediated embryonic mechanism.

5. LESSONS FROM THE "WAR ON CANCER"

The war on cancer, intended as a collective effort aiming at the reversal of the progressive increase of mortality caused by neoplasia, was declared nearly 5 decades ago. In spite of strenuous efforts in both basic and clinical studies aiming to expand our knowledge in cancer biology and therapy, and notwithstanding the considerable financial investment to support these studies, the unfolding of the war project failed to confirm the initial hopes and expectations (63,64). Generally, the decline in cancer mortality has been modest in recent years, and it is attributable primarily to early diagnosis due to improved screening technologies and preventive practices, rather than to the efficacy of new treatments (apart form some notable exceptions). On these grounds, there is now a consensus that long-term cancer-free responses are rare and a call for radically novel approaches in cancer therapy is required. It has been recently pointed out (65) that chemotherapy has not only failed to eradicate cancer but, paradoxically, can yield the opposite effect by "wiping out" the chemosensitive cells while favouring the clonal expansion of adapted chemoresistant cell populations. The authors proposed to shift from aggressive to milder clinical strategies with the goal to contain the tumor via cytostatic mechanisms rather than killing it using lethal cytotoxic therapies. Calling for a radical rethinking of current cancer treatments, Hanahan (66) suggested that therapeutic strategies should step back from the battle frontline against multiple targets in specific forms of cancer, to target fewer hallmarks shared by an ample array of cancers, so as to circumvent adaptive or evasive resistance.

According to our and other group's experience, a priori the RT-based anticancer therapy fulfils these recommendations for an innovative therapy, as the RT-dependent cancer-promoting mechanism:

1. is ubiquitously present in a variety of cancers, regardless of their histological origin, i.e. carcinoma, sarcoma, melanoma and haemathological malignancies. Therefore, RT is a common therapeutic target in a broad spectrum of human cancers;
2. provides cells with embryo-like plasticity, which constitutes a powerful source of adaptive resistance. Inhibition of RT restricts the plasticity and restores cell differentiation;
3. originates an aberrant expression profile for miRNAs, favouring in turn the emergence of a cancer-prone cellular environment. RT inhibition re-establishes the normal biogenesis of miRNAs and restores their regulatory control on cell transcriptome.

It remains to be seen whether LINE-1-encoded RT might represent a "universal target" for a cytostatic differentiation therapy against an ample spectrum of human cancers.

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