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# CCNG1 oncogene: a novel biomarker for cancer therapy /gene therapy

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## Abstract

**Background:** Metastatic cancer is associated with an invariably fatal outcome. However, DeltaRex-G, a tumor-targeted retrovector encoding a gene-edited dominant-negative *CCNG1* inhibitor gene, has induced long term (>10 years) survival of patients with chemo-resistant metastatic pancreatic adenocarcinoma, malignant peripheral nerve sheath tumor, osteosarcoma, B-cell lymphoma, and breast carcinoma.

**Objective:** To evaluate the level of *CCNG1* expression in tumors as a potential biomarker for *CCNG1* (Cyclin G1-blocking) inhibitor therapy.

**Methods:** *CCNG1* RNA expression levels that were previously measured as part of whole genome molecular profiling of tumors (TCGA, N=9161), adjacent "tissues" (TCGA, N=678) and GTEx normal tissues (N=7187) across 22 organ sites were analyzed. Differential expression of *CCNG1* and Ki-67 in primary (N= 9161) vs metastatic (N= 393) tumors were also compared in primary (N=103) vs. metastatic (N=367) skin cancers (i.e., melanoma).

**Statistical Analysis:** To detect systematically differential expression of *CCNG1* and Ki-67 expression between populations (e.g. tumor vs. normal), unpaired Student's t-tests were performed.

**Results:** Enhanced *CCNG1* RNA and Cyclin G1 protein expression were noted in tumors compared to normal analogous counterparts, and *CCNG1* expression correlated significantly with that of Ki-67. Moreover, *CCNG1* expression tended to be higher than that of Ki-67 in metastatic vs primary tumors.

**Conclusions:** Taken together with the emerging Cyclin G1 / Cdk / Myc / Mdm2 / p53 Axis governing *Cancer Stem Cell Competence*, this supportive data indicates: (1) *CCNG1* expression is frequently enhanced in tumors when compared to their normal analogous counterparts, (2) *CCNG1* and Ki-67 expressions are higher in metastatic vs primary tumors, (3) *CCNG1* expression is significantly correlated with that of Ki-67, and (4) *CCNG1* may actually be a stronger prognostic marker of stem cell competence, chemo-refractoriness, and EMT/metastasis than Ki-67. Phase 2 studies are planned to identify patients most likely to respond favorably to *CCNG1* inhibitor therapy.

**Keywords:** deltarex-g; human cyclin g1; cell cycle control; cancer gene therapy; oncogenic drivers; cancer stem cells; cell competence

## Introduction

Cyclins are a highly conserved family of executive regulatory proteins that govern the molecular-genetic "activation" of quiescent stem cells (i.e., *Cell Competence* to proliferate), as well as the definitive phases of cell cycle progression, in both normal tissue regeneration and in disease, through physical interaction with cognate cyclin-dependent proline-

directed protein kinase partners (CDKs) [1,2], which phosphorylate and regulate key regulatory substrates exerting cell cycle checkpoint control. The human Cyclin G proto-oncogene (*CCNG1*) was originally identified/cloned, silenced-experimentally, blocked-functionally (by gene-edited dominant-negative mutation), and characterized physiologically in the crucible of clinical oncology: thereby defining "**The pivotal Cyclin G1 / Cdk / Myc / Mdm2 / p53 Axis**" as a prospective *Unifying Theme* in oncology [3,4]—identifying Cyclin G1 expression profile as a frequent feature and key oncogenic driver of cancer stem cell survival [5], c-Myc oncogenicity [6], loss of DNA fidelity [7], cancer development [8], progression [9], epithelial-to-mesenchymal transition (EMT) [9], refractoriness to chemotherapies [10], and the troublesome immune anergy [11] of advanced metastatic cancers.

The *CCNG1* oncogene-a non-canonical cyclin exhibiting molecular aspects of both transcriptional regulation and cell cycle control [1,2] operates at the "first and rate-limiting step" in the animal cell cycle: that is, stem cell *Competence* ($G_0$ to $G_1$ boundary), where Cyclin G1/CDK activity phosphorylates and stabilizes the c-Myc oncogene/transcription factor [12-14], contributing to cancer progression and metastatic behavior [3-6]; while the physical association of Cyclin G1 with the Mdm2 oncogene, a ubiquitin ligase, is a negative regulator of the TP53 (p53) tumor suppressor: a guardian of DNA fidelity [7], which is commonly lost with cancer progression. As such, *CCNG1* has been clinically determined to be a strategic target for cancer gene therapy.

DeltaRex-G—previously developed clinically as Rexin-G [15-19]—is a tumor-targeted amphotropic MLV-based retroviral vector: (i) displaying a collagenous *Pathotropic Signature* (*SIG*)-binding/targeting peptide on its gene-edited gp70 envelope protein [20,21], and (ii) encoding a gene-edited dominant-negative expression construct of the *CCNG1* oncogene (dnG1)—the enforced expression of dnG1 *blocks* Cyclin G1-dependent cell survival function(s) and enforces apoptosis in a broad spectrum of cancer cells *in vitro*, in animal models, and clinically in advanced metastatic cancers [3,4] where notable safety, efficacy and long-term survival have been achieved (> 12 years) with repeated infusions of DeltaRex-G [22-24]. When administered intravenously, the tumor-targeted nanoparticles (~100 nm) actively seek-out and accumulate-in the tumor microenvironment (TME), binding with high-affinity to abnormal (anaplastic) *SIG* proteins exposed biochemically during tumor invasion, tumor angiogenesis, and/or reactive stroma formation [25]. Treatment with DeltaRex-G has demonstrated objective clinical benefits and prolonged survival in metastatic, chemotherapy-resistant pancreatic ductal adenocarcinoma, osteosarcoma, sporadic malignant peripheral nerve sheath tumor (sMPNST), and both hormone receptor positive and triple negative ductal carcinoma of breast [3,4, 22-24]. In light of the pivotal roles and survival function of Cyclin G1 in cancer stem cell biology [5-11], the observed clinical efficacy of DeltaRex-G, including long-term survival benefits achieved in advanced, metastatic, chemo-resistant cancers, suggests that DeltaRex-G may indeed be most efficacious and comparatively advantageous in advanced metastatic tumors that overexpress the pivotal and commanding *CCNG1* oncogene. Here, the aims of this investigation are to report augmented *CCNG1* oncogene expression in many solid tumors and provide the rational basis for profiling Cyclin G1expression in tumors to identify patients who are likely to benefit from *CCNG1* inhibitor therapy.

## Methods and Materials

All archived tumor samples are de-identified tissue samples previously used for other histopathologic examination and molecular profiling studies at NantOmics Bioinformatics Department of Nant and at the Cancer Center of Southern California/Sarcoma Oncology Research Center. Written consent was obtained from patients as part of histopathologic examination and molecular profiling of archived tumors as standard of care.

The Genotype-Tissue Expression (GTEx) project is an ongoing effort to build a comprehensive public resource to study tissue-specific gene expression and regulation. Samples were collected from 54 non-diseased tissue sites across nearly 1000 individuals, primarily for molecular assays including WGS, WES, and RNA-Seq. Remaining samples are available from the GTExBio bank. The GTEx Portal provides open access to data including gene expression, QTLs, and histology images.

### *CCNG1* RNA Sequence Analysis

Previously obtained whole-transcriptome expression levels across 22 commensurate organ sites within three different settings: tumor tissues (The Cancer Genome Atlas [TCGA], n=9161), tumor-adjacent tissues (TCGA, n=678) and normal tissues (Genotype-Tissue Expression Portal [GTEx], n=7187) were analyzed for *CCNG*1 expression.

### *Qualification of IHC assay for Cyclin G1 expression*

An anti-cyclin G1 IgG1 kappa light chain murine monoclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA, cat# sc-8016), coupled with MACH4 anti-mouse secondary antibody (BioCare Medical, Pacheco, CA) were used for immunohistochemical (IHC) analysis. The mouse monoclonal antibody was optimized on cell line tissue microarray and normal tissue microarray samples sectioned at approximately 4 μm and adhered to positively charged glass. Multiple antigen retrieval methods and a series of titrations of the cyclin G1 antibody were performed using the tissue microarray slides to determine the optimal antigen retrieval method and antibody concentration. Once the antibody concentration was chosen, accuracy, specificity, sensitivity, and the range of staining intensity was assessed using de-identified samples of hepatocellular carcinoma and breast carcinoma samples as positive controls, and normal tissues as negative controls. Additionally, three neoplastic tissues representing various degrees of cyclin G1 expression were chosen for an assessment of reproducibility and precision. Then, IHC analysis was performed on 27 de-identified formalin-fixed, deparaffinized samples, representing 13 types of cancer and 4 types of normal tissue. The optimized method is as follows: First, the samples were deparaffinized and rehydrated, and endogenous peroxidase activity was blocked. Then, antigen retrieval was performed in a decloaking chamber with Tris-EDTA for 20 minutes at 95° C. The anti-cyclin G1 antibody was applied at a dilution of 1:2000, and incubated for 30 minutes at room temperature, followed by application of the MACH4 mouse secondary antibody for 15 minutes at room temperature. A MACH4 HRP Polymer (BioCare Medical, Pacheco, CA) was then applied as the detection system, and incubated for 30 minutes at room temperature. The resulting product was incubated in chromogen DAB-beta (BioCare Medical, Pacheco, CA) for 10 minutes at room temperature, and was then counterstained with hematoxylin, dehydrated, cleared in xylene, and finally a coverslip was applied. The samples were then analyzed by a board-certified pathologist who scored the staining intensities of the samples on a scale of 0 to 3+. Reproducibility was tested in several ways: (1) intra-run reproducibility, with identical slides run in triplicate in one run on one day; (2) inter-run reproducibility with identical slides tested on three separate runs in three separate days; (3) inter-tech reproducibility, with identical slides run by two different technologists in two separate runs; and (4) inter-instrument reproducibility with identical slides run by the same technologist on two different instruments. Concordance factors were calculated based on the pathologist's scoring of the reproducibility slides. As an indirect confirmation of cyclin G1 assay specificity, additional IHC experiments were performed to compare expression of cyclin G1 to that of cellular proliferation marker Ki-67. Intra-run, inter-run, inter-tech, and inter-instrument reliability were all deemed as acceptable and *CCNG1* expression observed by IHC was in line with that predicted in the literature.

## Statistical analysis

To generate statistically comparable expression profiles for each sample, transcripts-per-million (TPM) values were generated using RSEM [26] scaled using only protein-coding transcripts, then converted to (approximately) Gaussian distributions by log2 transformation (i.e. log2[TPM+1]).

To detect systematically differential expression of *CCNG1* and Ki-67 expression between populations (e.g. tumor vs. normal), unpaired Student's t-tests were performed. t-tests were employed rather than non-parametric tests (e.g Wilcoxon test) as across tissues *CCNG1* and Ki-67 both displayed normal distributions (despite being skewed in some organ subsets) and non-parametric tests may be underpowered with low sample counts such as those provided in TCGA normal tissues. We evaluated cohort-level differential expression of *CCNG1* and Ki-67 in primary (n=9161) vs metastatic (n=393) across organ sites. Seeing as TCGA sources provide ample metastatic samples from skin cancer (e.g. melanoma) cases, we also performed primary vs. metastatic analysis within skin cancer specifically (n=103 and n=367, respectively). Additionally, we observed differential expression of *CCNG1* and Ki-67 between settings (primary and metastasis) within the same individuals. Matched pairs of primary and metastasic tumor samples were available for 28 patients across 12 organ sites in the TCGA cohort. Fold-change was calculated by taking the ratio of primary and metastatic log2-TPM values.

## Results

### Points to Consider #1: CCNG1is overexpressed throughout the TME, including adjacent non-tumorous areas.

Figure 1 shows the differential expression of *CCNG1* (x-axis) vs Ki-67 (y-axis) in tumor (green) and normal (blue and red) settings. There was no significant difference in *CCNG1*and Ki-67 expression across organ sites in TCGA tumor and adjacent non-tumor tissue within the TME (TCGA normal). In contrast, as shown in Table 1, *CCNG1* expression was significantly enhanced in TCGA tumor compared to normal tissue obtained post-mortem from patients with no tumors (GTEX normal). This is an important observation since proliferative tumor-associated microvasculature (TAM) and tumor-associated fibroblasts (TAFs) would also show enhanced expression of *CCNG1* and Ki-67 in the adjacent non-tumor tissue within the TME.

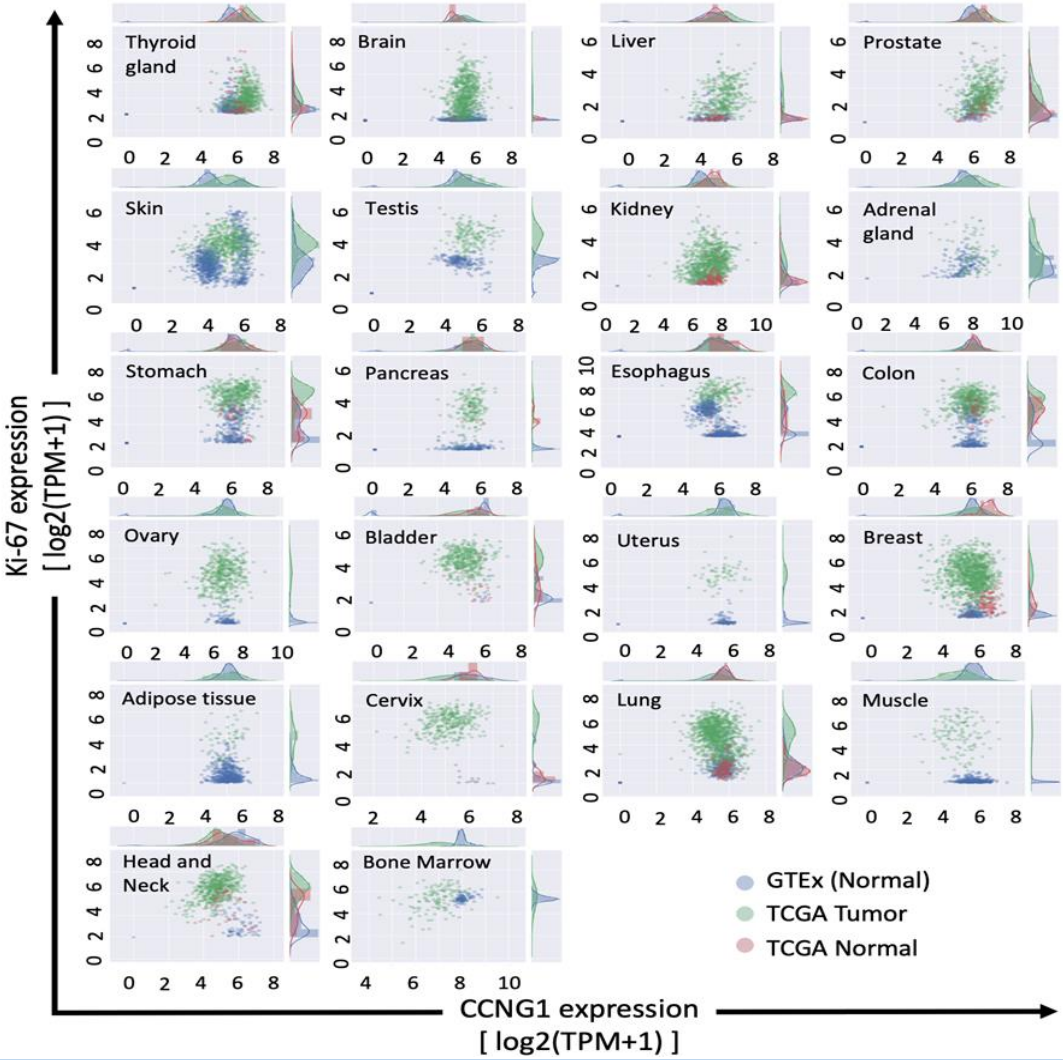


**Figure 1:** *CCNG1 expression across organ sites in normal and tumor tissues. Contrasted with Ki-67. Scatterplots of CCNG1 (x-axis) vs. Ki-67 (y-axis) for tumor (green) and normal (blue and red) settings. Plots are ordered by differential expression between TCGA tumor and GTEx normal. TCGA normal tissue has altered molecular expression status closer to the TCGA tumor and GTEX normal is distinctly different from both TCGA normal and TGCA tumor.*

| Tissue type | TCGA tumor vs. GTEX t-statistic | TCGA tumor vs. GTEX p-value | TCGA tumor vs. TCGA normal t-statistic | TCGA tumor vs TCGA normal p-value |
|---|---|---|---|---|
| Thyroid gland | 17.4053 | 1.82761e-58 | 5.77623 | 1.25923e-8 |
| Brain | 10.7032 | 4.58349e-26 | 2.78716 | 0.0054646 |
| Liver | 9.54118 | 5.73639e-20 | 4.66959 | 2.06486e-6 |
| Prostate | 9.42913 | 7.98963e-20 | 1.3379 | 0.181479 |
| Skin | 7.86357 | 7.2756e-15 | N/A | N/A |
| Testis | 6.05271 | 3.58683e-9 | N/A | N/A |
| Kidney | 5.98885 | 3.00984e-9 | -2.55981 | 0.0106152 |
| Adrenal gland | 5.37486 | 1.83838e-7 | N/A | N/A |
| Stomach | 4.52719 | 6.98267e-6 | -0.679563 | 0.497131 |
| Pancreas | 2.79005 | 0.0055392 | -0.566826 | 0.571535 |
| Esophagus | 1.70762 | 0.088022 | -1.48117 | 0.140167 |
| Colon | 1.53667 | 0.124732 | -2.77403 | 0.00572754 |
| Ovary | 0.466719 | 0.640887 | N/A | N/A |
| Bladder | 0.0242461 | 0.980668 | -3.07999 | 0.00220318 |
| Uterus | 0.00963925 | 0.992322 | N/A | N/A |
| Breast | -0.0198831 | 0.98414 | -12.4645 | 1.10424e-33 |
| Cervix | -4.08242 | 5.655564e-5 | -2.06655 | 0.039615 |
| Lung | -5.34723 | 1.03968e-7 | -6.02 | 2.34085e-9 |
| Head and neck region | -11.8523 | 3.22551e-29 | -4.97295 | 8.76887e-7 |
| Soft tissue, bone | -26.7181 | 1.5217e-87 | -1.18341 | 0.237721 |

**Table 1.** TCGA tumor versus GTEx normal CCNG1 RNA expression, and TCGA tumor versus TCGA tumor-adjacent normal tissue CCNG1 RNA expression

**Points to Consider #2: CCNG1expression is higher than that of Ki-67 in metastatic skin cancers (e.g., melanoma, basal cell carcinoma, squamous cell carcinoma).**

As shown in Figure 2 left panel, *CCNG1* and Ki-67 both showed enhanced expression in metastatic settings (orange) compared to primary tumors (green), but *CCNG1* outperformed Ki-67 specifically in metastatic skin cancer (right panel). Further, *CCNG1* expression was relatively higher in many metastatic samples than that of Ki-67 and did not track with Ki-67 (Figure 3). These data indicate that *CCNG1* oncogene expression may be a better indicator of metastasis than primary tumors.


**Figure 2:** *CCNG1 and Ki-67 both have increased expression in the metastatic setting, CCNG1 outperforms Ki-67 in skin cancer specifically. Violin plots showing distribution of normalized CCNG1 and Ki67 expression in primary (green) and metastatic (orange) settings across all TCGA (left) and the skin cancer subset (right).*

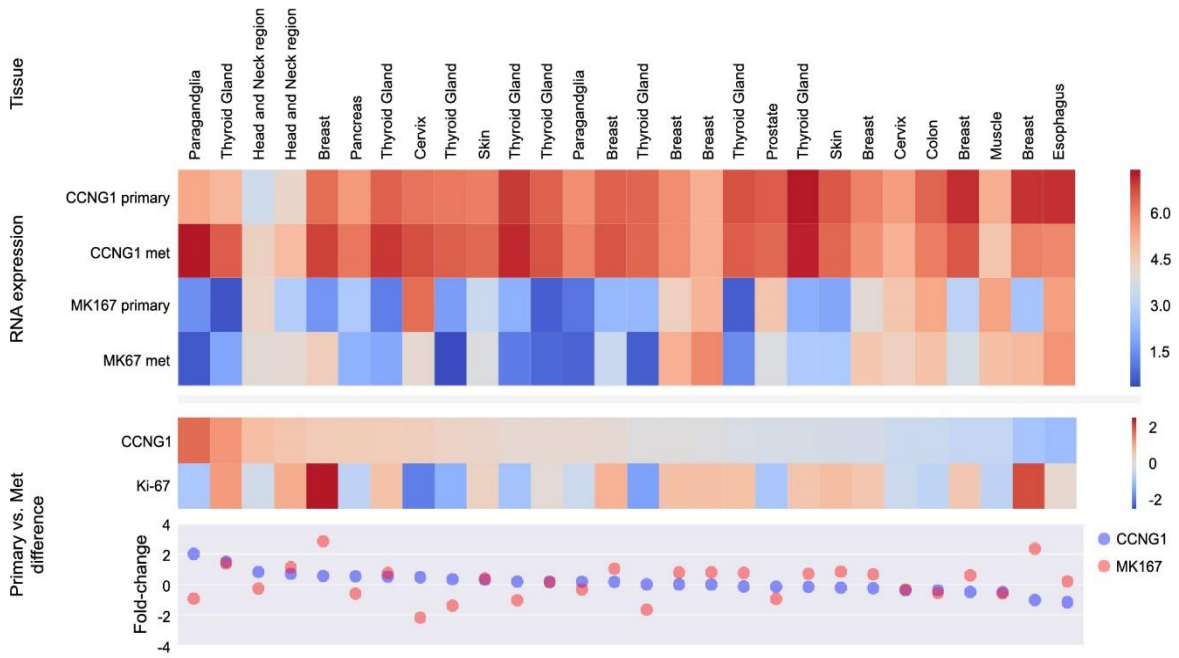


**Figure 3:** *CCNG1 may be an independent metastasis marker from Ki-67. Expression (top) and fold-changes (bottom) of CCNG1 and Ki-67 from 28 samples with both primary and metastatic samples in TCGA are shown. CCNG1 expression is relatively higher in many metastatic samples than that of Ki-67 and does not track with Ki-67.*

### Points to Consider #3: CCNG1 expression is enhanced in specific tumors by RNA sequence analysis.

As shown in Table 1, in 11 of 22 measured samples, *CCNG1* RNA expression was significantly overexpressed in TCGA tumor samples compared to GTEx normal tissues, including thyroid, brain, liver, prostate, skin, testis, kidney, adrenal, stomach, pancreas and esophageal cancers.

In 4 of the 22 samples, *CCNG1* RNA was under-expressed in the tumor samples, indicating that these tumors were in dormant or inactive state and as such, these tumors were not actively dividing, and may not be responsive to DeltaRex-G therapy, a retroviral based vector that integrates only in actively dividing cells [15-21].

### Points to Consider #4: CCNG1 nuclear protein expression is enhanced in most tumors examined by IHC.

Furthermore, IHC staining revealed significant cyclin G1 overexpression in almost all tumor samples and cancer cell lines (Table 2). For example, Figure 4 shows 80% nuclear *CCNG1*+ and nuclear Ki-67+ tumor cells in mesenchymal chondrosarcoma metastatic to brain.

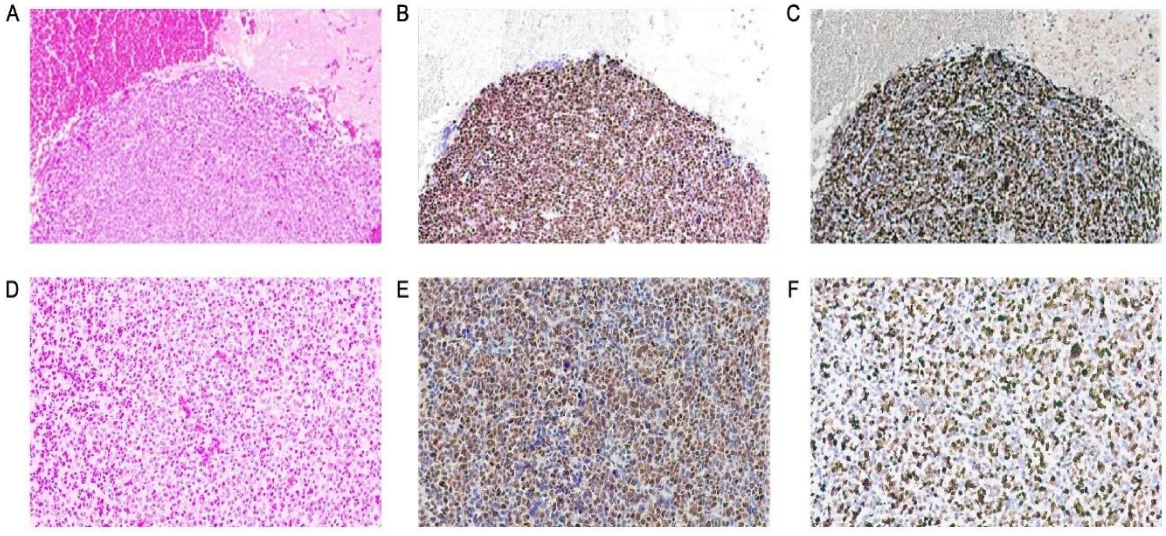


**Figure 4:** *Photomicrograph sections showing enhanced expression of CCNG1 nuclear protein (IHC staining) in mesenchymal chondrosarcoma metastatic to brain.From left to right, A&D (4X and 20X mag): H&E stain; B&E (4X and 20X mag): 80% CCNG1+ cancer cells; C&F (4X and 20X mag): 80% Ki-67+ cancer cells in biopsied specimen of chondrosarcoma metastatic to the brain.*

| Normal tissue | *CCNG1* nuclear staining percentage | Cancer cell line (tumor) | *CCNG1* nuclear staining percentage |
|---|---|---|---|
| Breast | 5% | Breast ILC (tumor) | 20% |
| | | Breast IDC (tumor) | 35% |
| | | Breast IDC (tumor) | 95% |
| | | Breast IC (tumor) | 60% |
| | | SK-BR3 breast carcinoma | 95% |
| Liver | 1% | HCC (tumor) | 90% |
| | | HCC (tumor) | 40% |
| | | HCC (tumor) | 5% |
| | | HCC (tumor) | 0% |
| Lung | 0% | HCC-78 NSCLC | 90% |
| Brain, cerebellum | 0% | T98G glioblastoma | 100% |
| Tongue | 0% | Sarcoma NOS (tumor) | 70% |
| Skeletal muscle | 0% | Leiomyosarcoma (tumor) | 30% |
| | | Angiosarcoma (tumor) | 90% |
| | | Liposarcoma (tumor) | 40% |
| | | Chondrosarcoma (tumor) primary tumor | 0% |
| | | Chondrosarcoma (tumor) metastatic to brain | 80% |
| Colon epithelium | 30% | Colorectal carcinoma (tumor) metastatic to liver | 90% |
| | | Colorectal carcinoma (tumor) metastatic to liver | 20% |
| None | | HDLM2 Hodgkin lymphoma | 95% |
| None | | Jurkat T lymphoblastic leukemia | 95% |
| None | | Karpas ALL | 100% |
| None | | Jeko-1 mantle cell lymphoma | 95% |

**Table 2.** *Summary of cyclin G1 expression, as ascertained by IHC, for normal vs cancerous tissues.*

## Discussion

During embryological development, the *CCNG1* (Cyclin G1) oncogene product normally cooperates with the TP53 (p53) family of tumor suppressor proteins in mediating genome stability: operating at multiple cell cycle checkpoints in proliferative somatic cells [27-30]. Characterization of the *CCNG1* oncogene in terms of clinical oncology (dysregulated expression, viral subversion, oncogenic interactions, and stem cell survival functions) revealed Cyclin G1 also complexes with the c-Myc and Mdm2/Hdm2 oncogenes—thus identifying the pivotal ***Cyclin G1 / Cdk / Myc / Mdm2 / p53 Axis*** of ***cancer stem cell competence*** as a strategic target for broad-spectrum cancer gene therapy [3,4] and a potentially unifying theme of cancer ontogeny (*Cyclin G1-complex*), EMT, and metastatic progression.

Reimer et al. [31] was among the first to describe histopathological overexpression of Cyclin G1, with subcellular localization at DNA replication foci, suggesting potential diagnostic and prognostic implications of *CCNG1*dysregulation in both breast and prostate cancer cells *in vitro*, as well as cancer cells *in situ* from tumor specimens, using differential display polymerase chain reaction (PCR) screening [31]. Following DNA damage, Reimer et al. showed that Cyclin G1 was triggered to cluster in a discrete nuclear pattern at DNA replication foci; demonstrating subcellular co-localizing at foci containing the replication-associated proliferating cell nuclear antigen (PCNA), which physically binds to the classic CDKN1A/ $p21^{Waf1/CIP1}$-tumor suppressor [32], to regulate genomic stability, cell cycle arrest, and senescence in response to DNA damage through myriad complex physical interaction(s) with TP53 (p53)[7,33].

In 2015, Jiang et al. [34] advanced the clinical understanding of *CCNG1* by investigating its molecular expression and biochemical role in governing cellular proliferation and apoptosis of epithelial ovarian cancer (EOC), where *CCNG1* (Cyclin G1) was found to be upregulated in EOC tumors, compared with normal ovarian tissues. In EOC, *CCNG1* expression was closely correlated with differentiation grade (P = 0.009) and malignant tumor cells in ascites (P = 0.009). The Kaplan-Meier curves showed that higher expression of *CCNG1* was associated with significantly shorter survival in EOC patients, while multivariate analysis suggested *CCNG1* expression is an independent prognostic factor in terms of overall survival. Combined immunofluorescence and flow cytometry analysis confirmed that silencing of *CCNG1* with shRNA is lethal to EOC: promoting apoptosis of ovarian cancer cells. Taken together, Jiang et al.'s findings suggest that *CCNG1* may indeed serve in the prognosis of EOC patients, as well as a prospective therapeutic target.

Likewise, a molecular histological evaluation of *CCNG1* expression in colorectal neoplasia, demonstrated *CCNG1* overexpression in 91% of colorectal tumors studied [35]. The Cyclin G1-positive cancer patients were evenly distributed between men and women, and between tumor locations and grading. Moreover, this comparative study noted that Cyclin G1 overexpression was a more frequent event than the commonly dysregulated *CCND1/PRAD1/ Cyclin D1* proto-oncogene (found in only 42% of colorectal adenocarcinomas) [35]; thereby suggesting an enhanced therapeutic potential for *CCNG1*/Cyclin G1 oncogene as a molecular-genetic marker and a postulated target for treating colorectal neoplasias.

In 2012, Russell et al. [36] reported that Cyclin G1 regulates the outcome of taxane-induced mitotic checkpoint arrest demonstrating that *CCNG1* amplification promotes "cell survival" after paclitaxel exposure in ovarian cancer cells. Conversely, *CCNG1* depletion by RNA interference enhanced paclitaxel-induced cell death via apoptosis. Further, *CCNG1* amplification was associated with significantly shorter post-surgical survival in patients with ovarian cancer who had received adjuvant chemotherapy with taxanes and platinum compounds [36]. Indeed,

***CCNG1* overexpression *per se* was determined to promote epithelial-mesenchymal transition (EMT) to an aggressive "metastatic" phenotype,** which is mechanistically associated with chemotherapy resistance in gastric, liver, ovarian and other cancer types [8-10, 37].

The mechanisms of *oncogene addiction* and cancer progression include loss of natural *tumor suppression* focused on *CCNG1*/Cyclin G1 proto-oncogene: in 2007, a series of high-throughput screens investigating the role of microRNAs in human hepatocellular carcinoma (HCC) identified miR-122 as the leading species of microRNA that was either lost or significantly down-regulated in about 70% of HCCs, and in all of the HCC cell lines [38]. Notably, these studies demonstrated *CCNG1* as a gene target of miR-122, due to the inverse relationship between miR-122 and *CCNG1* expression seen in HCC. Loss of miR-122 gene suppression and consequential *CCNG1* overexpression was associated with increased proliferation of HCC cells, disease progression, and metastasis [39], while re-expression of miR-122 was shown to inhibit both their tumorigenic properties [40] and metastatic potential [9,41]. It was confirmed that, by controlling *CCNG1* expression, miR-122 impacted the stability and transcriptional activity of p53, as restored miR-122 expression reduces the metastatic invasiveness of HCC-derived cell lines [41]. Moreover, the inhibitory effect of experimentally restored miR-122 expression on *CCNG1* levels increased the sensitivity of HCC cells to both sorafenib [40] and doxorubicin [41], thereby establishing a mechanistic basis for the future development of combined chemotherapy and miR-122 mimetic and/or CCNG1-based cancer therapies.

This is the first study that uses bioinformatics and a qualified IHC protocol to study a large number of tumors for *CCNG1* expression compared to their analogous counterparts. As cancer treatment continues to trend toward more highly directed molecular therapies, efforts have been made to determine how to best assess which patients may benefit from specific targeted therapies. Cyclin G1 dysregulation is an important negative regulator of p53 activity, allowing for disinhibited cell cycle progression with decreasing DNA fidelity [3,4]. In the present study, we found that Cyclin G1 is overexpressed in many tumor samples; and that in metastatic tumors, Cyclin G1 expression is higher than that of the Ki-67 labeling index of cell proliferation (an important prognostic factor), providing mechanistic support for a Cyclin G1-centric view of cancer stem cell *Competence*, as distinguishable, in terms of aligning targets with prospective therapies, from the enzymatic and biochemical phases of proliferative cell cycle control. Together with the broad clinical efficacy of CCNG/Cyclin G1 inhibitor (DeltaRex-G) therapy, this finding supports the emerging focus on the Cyclin G1 oncogene [3,4], which should be included in the molecular-genetic profiling of tumors at diagnosis and in post-treatment tumor specimens.

DeltaRex-G is the first and, so far, only tumor-targeted retrovector encoding a *CCNG1* inhibitor gene [15-22], expressed therapeutically as a gene-edited dominant-negative (dnG1), i.e., a cytotoxic Cyclin G1 pathway-blocking construct [3,4]. DeltaRex-G has been found to be of significant and durable clinical benefit in an array of advanced metastatic cancers, as salvage therapy [19-25]; therefore, the ability to screen and identify patients with specific overexpression of the commanding *CCNG1* proto-oncogene can help inform clinicians as to which patients might benefit the most from *CCNG1* inhibitor therapy. Additionally, finding that *CCNG1* expression varied over time in some tumors suggests that genomic data from multiple biopsies or from circulating cell-free tumor DNA may be additionally informative, in relation to predicting the optimal timing and duration of DeltaRex-G and other future *CCNG1 gene-based* cancer therapies.

Our findings in clinical tumors—that Cyclin G1 is over expressed in tumor cells at metastatic sites when compared to primary tumors—is in agreement with clinicopathological analysis in HCC [9], wherein the pivotal role of CCNG1/Cyclin G1 expression in cancer progression is viewed as a novel prognostic biomarker and prospective therapeutic target for the clinical management of metastatic disease. One important pathway driving the epithelial-mesenchymal transition (EMT) of metastatic cancers is the stabilization of the "Snail-family" of transcription factors, described as a molecular EMT "switch" to a motile and aggressive phenotype characterized by increased invasive potential, cytoskeletal reorganization, and molecular-genetic down regulation of E-cadherin [9,42], required for maintenance of epithelial architecture. Biochemically, GSK-3β kinase phosphorylates Snail, a transcriptional repressor involved in negative regulation of E-cadherin and a marker of malignancy [43,44]. Conceptually, the Cyclin G1 Axis promotes EMT and cancer metastasis, in part, via activation of the PI3K/Akt/ GSK-3β/Snail-dependent pathway, suggesting that two parameters: Akt-activation in correlation with elevated Cyclin G1 levels may be a more powerful predictor of a poor prognosis [9,45].

This study also found *CCNG1*/Cyclin G1 to be overexpressed, not just in flagrant tumor cells, but within the supportive neovascular and stromal cells of the tumor microenvironment (TME) as well. The term "angiogenic switch" describes the point in tumor progression when pro-angiogenic factors begin to predominate over anti-angiogenic factors, leading to vascular proliferation in the TME, allowing the tumor to receive nutrients required to progress and to maintain its high energy demand [46,47]. Additionally, TME vasculature encourages evasion of antitumor immune surveillance, in part by impeding lymphocyte-endothelial cell interaction and upregulating immunosuppressive leukocytes [11,47]. The specific cytocidal activity of DeltaRex-G in human tumors, including proliferative tumor-associated TAMs and TAFs suggests that DeltaRex-G breaks anergy by enhancing immune cell trafficking within the TME [24]. Moreover, localized GMCSF-immune stimulation, in combination with Cyclin G1 blockade, has demonstrated promising clinical results and survival value in advanced metastatic cancers [21], while chemo-sensitization, has been experimentally demonstrated in the medical literature [40,41]. Taken together with the notable therapeutic efficacy and long-term survival milestones achieved in advanced cancers with tumor-targeted dnG1 (DeltaRex-G) [3-4,22-23,48], the pivotal and commanding role of the *CCNG1* oncogene (Cyclin G1/Cdk/Myc/Mdm2/p53 Axis) of cancer stem cell *Competence* provides a unifying perspective for advancing new combinatorial and molecular approaches to cancer management [4,24,48-50]. Phase 2/3 studies are planned to identify patients who are likely to respond favorably and/or benefit most with DeltaRex-G gene therapy by correlating *CCNG1* expression levels and treatment outcome parameters in cancer patients treated with DeltaRex-G with or without chemotherapy, targeted therapy and/or immunotherapy, revealed in the profile of oncogene expression.

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