Cytogenetic Exploration of Ewing Sarcoma with Emphasis on Variant Translocations: A 12-Year Study at a Regional Cancer Centre in South Asia

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Abstract

Background: The diagnosis of Ewing sarcoma (ES) is challenging in the absence of tumour specific t (11;22), especially if there are overlapping morphological and immunohistochemical features. Knowledge of variant translocations and additional chromosomal abnormalities helps in diagnosis and prognostication. The present study provides an overview of the cytogenetic abnormalities associated with ES to construct a classification model for cytogenetic work up of the disease.

Materials & Methods: The retrospective exploratory study considered clinical data procured from patient files related to ES diagnosis between January 2007 and December 2018.

Results: Two hundred diagnostically confirmed cases of ES were cytogenetically categorized into classical, non-classical and miscellaneous groups, based on the rearrangement of 22q12 in metaphases. The classical group comprised 125 patients with specific t (11;22). The main focus of the study was on non-classical group having variant translocations (24 patients) or derivative chromosome (22q12) as sole abnormality (15 cases) in metaphases. The diagnosis in this group was concluded after corroborating the cytogenetic findings through other diagnostic tests. The miscellaneous group included patients with normal karyotype (eight cases), one case of t(1;16) as sole abnormality and those having polyploid metaphases (27 cases) with marker chromosomes. Clinical significance of variant translocations, ploidy status and numerical abnormalities has been discussed.

Conclusion: The overall frequency of variant translocation in ES is 12%. Chromosome 12 has been identified as the second most legitimate partner of chromosome 22q12. Additional numerical abnormalities associated with variant translocations did not differ significantly from that of classical translocations. Further investigations are warranted to identify the putative genes of new variant translocations, which may assist in better understanding the pathogenesis of ES and to define therapeutic molecular targets.

Keywords: Ewing sarcoma; Variant translocation

Key messages: Morphological diagnosis of ES is challenging, especially if the immunohistochemistry is non-contributory. Cytogenetics is the fundamental investigation in routine diagnosis of ES based on t(11;22). Recent years have witnessed significant interest in understanding variant translocations of ES due to its presentation at unusual sites and its prognostic significance. The present study has designed a cytogenetic categorization model for diagnosis and prognosis of ES. Chromosome 12 has been identified as the second most legitimate partner of 22q12. The identification of newer translocation partners requires genetic exploration for better understanding the ES pathogenesis.

Introduction

Ewing Sarcoma (ES) is the most common bone tumour in children and the third most common primary malignant tumour in adults[1,2]. It is the first sarcoma to be defined by a specific chromosomal translocation[3]. Due to considerable overlap in clinical and histomorphological features of ES with other small round cell tumours, cytogenetics and molecular techniques have been proven to be indispensable diagnostic tools[4-6].

Approximately 90% of ESs are characterized by t(11;22)(q24;q12) resulting in tumour-specific chimeric EWSR1-FLI1 fusion gene encoding for an oncogenic transcription factor[4,7,8]. Variant translocations occur in less than 10%, in which EWSR1 gene on 22q12 fuses with genes on chromosomes other than 11[1,4,7]. The progression and aggressive nature of the disease can be related to secondary chromosomal abnormalities such as trisomy 8, trisomy 12 and t(1;16)[9-11].

Cytogenetics and molecular techniques have contributed significantly to the characterization of malignant neoplasms. There is paucity of literature evidence on variant translocations in ES, and most of them are associated with extra-osseous presentation. Cytogenetics serves both as a diagnostic technique and a valuable prognostic indicator. It evades the need of biopsy for histopathology and immunohistochemistry (IHC), as it involves the collection of fine needle aspiration (FNA) sample through a minimally invasive procedure. The present is intended to provide a comprehensive overview of chromosomal abnormalities related to ES, with review of literature emphasizing variant translocations.

Subjects and Methods

The retrospective exploratory study considered diagnostically confirmed 335 cases of Ewing's sarcoma referred from the departments of Pediatric Oncology, Medical Oncology and Surgical Oncology between January 2007 and December 2018. Clinical data were procured from patient files. FNA samples were obtained according to standard medical procedure and the cells were suspended in RPMI-1640 medium, supplemented with 15% qualified, heat-inactivated fetal bovine serum (GIBCO). Eighteen-hour overnight culture was constituted by incubating the cell suspension in culture medium at 37^0C. This was followed by mitotic arrest with Karyomax-Colcemid in 0.05 μg/ml concentration and incubation of the cultured sample at 37^0C for 30 minutes. Cells were subjected to hypotonic treatment with potassium chloride (0.075M) and incubation of sample for another 30 minutes at 37^0C. This was followed by fixation in Cornoy's fixative solution (methanol and acetic acid in 3:1 concentration) and GTG-banding of the prepared slides. Chromosomal analysis and complete karyotyping were done in accordance with the International System for Human Cytogenomic Nomenclature (2005-2016) guidelines.

Statistical analysis

Descriptive statistical analyses (frequencies and means) were carried out for baseline clinical and cytogenetic parameters.

Results

Culturing was successful in 200 out of 335 cases (59.7%). The selected cases were cytogenetically categorized into classical, non-classical and miscellaneous groups based on the rearrangement of chromosome 22q12 in metaphases (Table 1). In the classical group comprising of 125 patients, the diagnosis of ES was concluded on the basis of t(11;22)(q24;q12). The non-classical group was subdivided into: i) simple variant translocations (V;22q12) (12 cases; 6%), wherein 22q12 translocated with a different chromosome other than 11; ii) complex variant translocations (V;11;22q12) (12 cases; 6%) characterized by translocation of 22q12 with a third chromosome along with chromosome 11 (Fig.1). iii) and der (22)t(?;22)(?;q12) translocation as a sole abnormality(15 patients; 7.5%). In the non-classical group with variant translocations, the diagnosis was confirmed by morphology, IHC and fluorescent *in situ* hybridization (FISH). FISH diagnosis of ES was rendered based on the typical signal pattern using break apart probe for EWSR1 gene (Fig.2). The miscellaneous group was defined by the presence of normal chromosome 22 in metaphases. It included normal karyotype (eight cases; 4%), polyploid metaphases with marker chromosomes (27 cases; 13.5%), and one case with der(16)t(1;16)(q23;p13) translocation as a sole abnormality. In the miscellaneous group, the diagnosis was concluded mainly based on morphology, IHC and FISH.
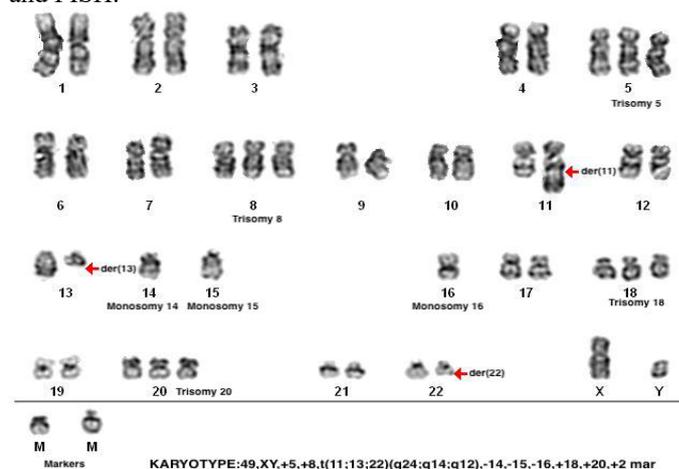

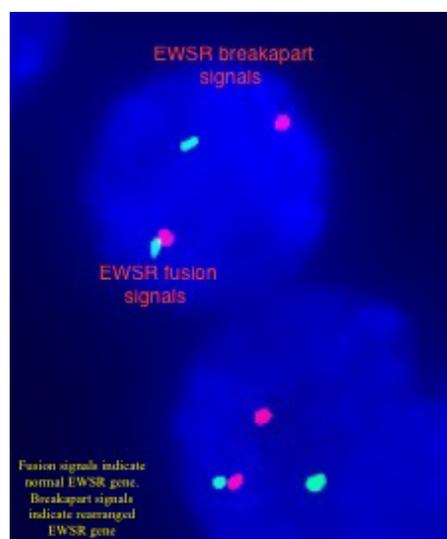KARYOTYPE:49,XY,+5,+8,t(11;13;22)(q24;q14;q12),-14,-15,-16,+18,+20,+2 mar

Figure 1:

Figure 2:

Table 1: Cytogenetic Categorization of Ewing Sarcoma based on Chromosomal Abnormalities

Classical group of ES with tumor specific t(11;22)	Non-Classical group of ES 1. Simple Variant Translocation t(V;22) 2. Complex Variant Translocation t(V;11;22) 3. der(22)t(?;22)(?;q12)	Miscellaneous Group (Normal Chromosome 22 in metaphases) 1. Normal Karyotype 2. t(1;16) 3. Polyploid metaphases with marker chromosomes
ES: Ewing sarcoma. V: Variant Chromosome involved in Variant Translocation		

The overall frequency of additional numerical and structural abnormalities noted was 47% (94/200). Whole chromosome gains were more common than losses (Fig.3). Around 62% (78/125) of the ES cases with classical translocation had additional numerical abnormalities. The most common translocations noted were trisomies of chromosomes 8 and 12, followed by trisomies of 5, 2 and 14. The frequency of additional structural abnormalities in ES with classical translocation was 36% (45/125). Most common among these were t(1;16) and structural abnormalities of chromosome 1. Nearly 67% (16/24 cases) of the ES with variant translocations had additional chromosomal abnormalities (Tables 2 & 3). The commonly noted abnormalities were trisomy 8and 7, followed by trisomies of 4 and 12. Additional structural abnormalities were diverse. The ploidy status was assessed in all patients. Forty-five percentage (56/125) of ES with classical translocation had hyperdiploidy, while it was observed in 67% (16/24) of ES with variant translocations.
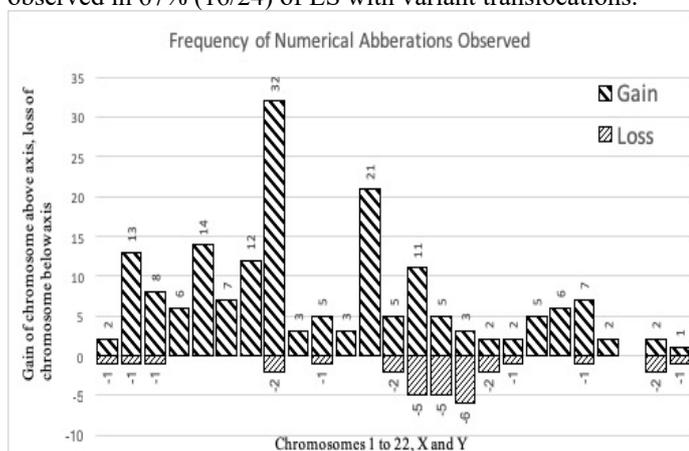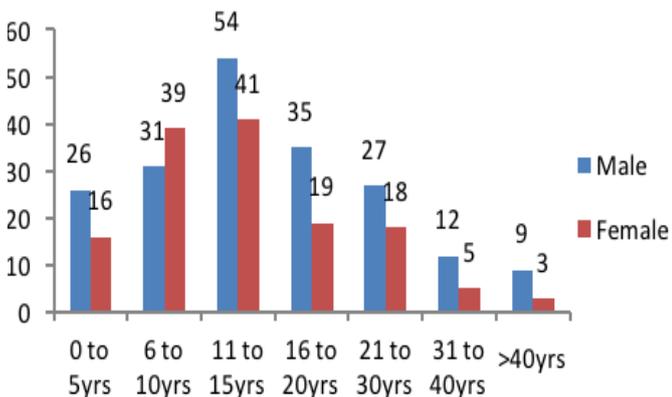

Figure 3:

Figure 4:

Table 2: ES with Simple Variant Translocation (V;12)

Variant Chromosomes & their breakpoints	karyotype	Age	Sex
12q12	46,XX,t(12;22)(q12;q12)	4yrs	F
12q13	46,XX,t(12;22)(q13;q12)	13yrs	M
12p13	46,XY,t(12;22)(p13;q12)	21yrs	M
12q24	47,XY,t(12;22)(q24;q12),+mar	22yrs	M
12q24	57,X,+1,+6,+7,+8, +8,+9,+11,t(12;22) (q24;q22),+20,+20,+20,+20	12yrs	M
12q24	48,XY,+4,t(6;7) (q11;q11),+8,t(12;22) (q24;q12),-16,+22	22yrs	M
21q22	47,XY,+3,del(6)(q24),t(21;22) (q22;q11)	5yrs	M
21q22	47,XX,+X,-10,t(21;21;22) (q22;q22;q12),+mar	7yrs	F
20q13	47,XX,+19,t(20;22)(q13;q12)	26yrs	F
17p13	47,XX,t(17;22)(p13;q12),+mar	5yrs	F
4q35	46,XX,t(4;22)(q35;q12)	12yrs	F
22q12	51,XY,+4,+7,+8,+9,+21,t(22;22) (q13;q12)	18yrs	M

The frequency of ES, according to age and gender, are depicted in figure 4. The corresponding percentages of affected males and females were 58% (194/335 cases) and 42% (141/335 cases), and the respective median age of occurrence noted were15 and 16 years. Details of tumour site were available for 108 patients and the most common site of involvement was skeletal. It included bones of lower extremities (28/108), pelvis (26/108), ribs (15/108), upper extremities (10/108), scapula (8/108), clavicle (3/108), and three mandibular, three spinal, two nasal and one skull lesions. Extra skeletal involvement included soft tissues of chest wall, scalp, thigh, arm, mediastinum, retroperitoneum, kidney and pancreas.

Discussion

EWS-ETS chromosome rearrangements are seen in bone and soft tissue tumourssuch as Ewing sarcoma, neuro epithelioma, peripheral primitive neuroectodermal tumour (pPNET) and Askin tumour.ES is categorized as small round cell tumour[12] and can be differentiated from other small round tumour cells by the recognition of non-random t(11;22)(q24;q12) chromosome rearrangement[13]. The EWSR1 with FLI1 or ERG fusions are well demonstrated, whereas the FUS-ERG fusions are very rare. It is highly challenging to diagnose ES with EWSR1 FISH assay alone since EWSR1 rearrangement may be seen in many other tumors such as desmoplastic small round cell tumor, clear cell sarcoma and myxoid liposarcoma. Additional tests such as ERG FISH or RT-PCR/ next generation sequencing should be employed for more result-oriented diagnosis[14]. However, unlike FISH or RT-PCR, cytogenetics provides global assessment of chromosomal abnormalities without the need for prior knowledge of histological diagnosis. In addition to primary abnormality, secondary chromosomal changes can be detected which possibly have role in clinical course of the disease.

TET-ETS translocations result in large number of oncological gene rearrangements and EWS protein plays a major role in ES. ETS consists of many homogenous proteins involved in various translocation process. Some of the identified genes and proteins belonging to the PEA3 subfamily are ETV1, ETV4 and ETV5 proteins. The translocations affecting ETV1 or ETV4 in Ewing tumours are the causes for carcinogenesis. Similarly, translocations involving ETV1, ETV4 and ETV5 are responsible for prostate cancer. Protein fusions are useful for the characterization or identification of the type and origin of sarcomas. Ewing tumour translocations mainly involve FLI1, ERG, FEV, ETV1 or ETV4 genes and rarely EWS-ETS fusions[15]. There are studies on impairing the growth of ES tumours by controlling the upregulation process[16].

The current study portrays a catalogue of chromosomal abnormalities in ES and summarizes findings of systematic analysis of these abnormalities with clinical correlation and in-depth review of literature. Clinical evaluation revealed that males were more frequently affected than females (57.9% vs. 42% respectively) and the peak incidence of the disease was in the age group of 11-15 years. These findings were in concordance with other studies[2]. ES occurs in all the age groups from infants to elderly, though majority are seen in < 20 years of age[4]. In the present study, 78% (261/335) the subjects had age < 20years. The current study noted decline in the incidence of the disease with advancing age. Only 12 patients (4%) were > 40 years of age. Extra skeletal lesions and metastatic disease are more common in older patients having poor survival rate[19]. Detailed clinical information was available for five patients of > 40years of age, and two of them had extra-skeletal ES and stage IV disease at diagnosis.

The most common site of involvement noted in the current study was skeletal, comprising of bones of lower extremities, pelvis, flat bones of chest, upper extremities, scapula, clavicle, and bones of head and neck in the decreasing order of frequency. Three mandibular, two nasal and one skull lesions were found. ES of head and neck is rare and is associated with improved prognosis due to smaller size of the tumour and earlier detection. Extra-osseous ES may occur in the soft tissues or visceral organs. Fifteen patients in the present study had extra-skeletal lesions, most commonly arising in soft tissues, and six of them had metastatic presentation. Most reported variant translocations of ES in literature were from extraskeletal sites. Except one mediastinal lesion with simple variant t(21;22), all extra-osseous lesions had classical t(11;22) and four of them were accompanied by additional numerical abnormalities. Two paediatric patients had ES of kidney and one of them had stage IV disease at the time of diagnosis. One patient with pancreatic lesion had hyper diploid karyotype with trisomies of chromosomes 3, 8 and 7, in addition to translocation t(11;22). Visceral ES is very rare, with only few reported cases of renal and pancreatic disease till date.

Table 3: ES with Complex Variant Translocation (V;11;22)

The third chromosome & the-breakpoints	Karyotype	Age	Sex
1p22, 14q11	47,XY,t(1;11;14;22)(p22;q24;q11;q12),+4,+8,-13,-14,+mar	8yrs	M
1p32	53,XX,t(1;11;22)(p32;q24;q12),+del(1)(p32),+2,+8,+12,+20,+21	25yrs	F
1q21	79,XY,<3n>,+X, +Y,-1,-1,t(1;11;22)(q21;q24;q12)x2,+2,-8,-13,+14,+2mar	7yrs	M
1q31	48,XY,t(1;11;22)(q31;q24;q12),+2mar	3yrs	M
2p13	46,XX,t(2;11;22)(p13;q24;q12)	14yrs	F
5q31	50,XX,del(1)(p22)x2,+5,+7,t(5;11;22)(q31;q24;q12),+12,+mar	18yrs	F
9p12, 12q24	46,XX,t(9;11;12;22)(p12;q24;q24;q12),add(9)(q34)	14yrs	M
9q34	46,XY,t(9;11;22)(q34;q24;q12)	29yrs	M
V	50,XY,+8,t(V;11;22)(?;q24;q12),+13,+2mar	6yrs	M
13q14	49,XY,+5,+8,t(11;13;22)(q24;q14;q12),-14,-15,-16,+18,+20,+2 mar	18yrs	M
V	49,XY,del(7)(q22),+8,add(9)(p23),t(11;V;22)(q24;?;q12),+2mar	45yrs	M
V	46,XX,t(11;V;22)(q24;?;q12)	26yrs	F
V: refers to unidentified Variant chromosome. ?: refers to uncertain breakpoint			

The success rate (59.7%) noted in the current study in achieving adequate chromosome preparation by cytogenetic technique was in par with other studies[8,9]. The translocation t(11;22)(q24;q12) is highly specific for EFT (Ewing Family Tumors), since it has been ruled out by different genetic and molecular methods in different morphologically identical tumours such as neuroblastomas, giant cell tumours and rhabdomyo sarcoma[6,7]. The study demonstrated t(11;22) in 62.5% of patients by conventional cytogenetics. Overall incidence of variant translocations noted was 12%. Provisional diagnosis of ES was made in case of der(16)t(1;16)(q23;p13) as a sole abnormality. t(1;16) is the second most common non-random translocation found in ES[8,9] and it serves as a potential surrogate marker for the disease diagnosis, in the absence of chromosome 22q12 rearrangement on cytogenetics. Normal karyotype and polyploid metaphases with marker chromosomes represent cryptic 22q12 (EWSR1 gene) rearrangement, which has been proven only in the recent years by IHC and FISH.

Myriad of variant translocations in ES result in the fusion of either EWSR1 gene on 22q12 or homologous FUS gene on 16p11 with ETS or non-ETS family of transcription factors[6-8]. About 30 genes are present in ETS family of transcription factors including FLI1 on 11q24 and other genes that take part in variant translocations such as ERG on 21q22, ETV1 on 7p22, FEV on 2q33 and ETV4 on 17q12[2,3]. EWSR1 gene fusion with non-ETS family of genes, such as NFATc2 on 20q13, SP3

on 2q31 and SMARCA5 on 4q31, may occur in a subset of un-differentiated round cell tumours with ES-like morphology[6,7].

There are anecdotal reports of variant translocations in the literature. In the current study, 12 (6%) cases of ES had simple variant translocations. Chromosome 21 has been document-ed in the literature as the most common chromosome involved in variant translocations[7,21]. In contrast, the present study showed chromosome 12 with varying break points as the second most common translocation partner of 22q12. Involvement of 12q24 was observed in four cases. This breakpoint is implicated in the pathogenesis of many cancers. 12q13 was involved in two patients in the current study and one of them had aggressive disease with the development of paraplegia. t(12;22)(q13;q12) with different fusion genes have been identified in many soft tissue sarcomas[6]. The researchers observed t(12;22)(p13;q12) in a 13-year-old boy with a locally advanced disease in sacral region. t(12;22)(p13;q12) has been documented very rarely in ES[16]. The putative genes noted at different breakpoints of chromosome 12 in variant translocations require further exploration.

Desmaze C *et al.*(1997) and Maire G et al.(2008) have demonstrated rearrangement of 21q22 in non-specific and cryptic complex translocations[21]. t(21;22)(q22;q12) is considered as the most common variant translocation, resulting in EWS-ERG fusion gene[6,7,21,23]. The present study reported two cases of variant translocation with Ch 21q22 involvement: one simple variant t(21;22) with mediastinal lesion and another unique complex variant translocation with complex rearrangement of both copies of chromosome 21 having normal chromosome 11. It has been presumed that this might involve dual copies of ERG gene influencing the biology and clinical nature of the disease. However, this needs further confirmation through molecular workup and close clinical follow-up.

Chromosome 4 has been implicated in a subset of primitive round cell sarcomas with overlapping features of Ewing's (Ewing-like) sarcoma[6,24]. Some examples are t(4;19)(q35;q13) with CIC-DUX4 fusion and t(4;22)(q31;q12) with EWSR1-SMARCA5 fusion[6,24]. DUX4 at 4q35 could be the pathogenic partner gene in the current case of ES with t(4;22)(q35;q12), due to involvement of similar breakpoint on chromosome 4 in Ewing-like tumours.

t(20;22)(q13;q12) reported in this study, is another rare example of simple variant translocation, wherein EWSR1 gene couples with non-ETS family of genes such as NFATc2[7,22]. The present study has reported the first case of an18- year-old boy with t(22;22)(q13;q12), accompanied by additional numerical abnormalities.

Complex variant translocation involving a third chromosome, in addition to chromosomes 11 and 22, is a rare entity and very few cases are reported to date. The current study identified 12 such cases. Though the third partner chromosome and their breakpoints were varied, chromosome 1 appeared to be the most consistent one (41.6%; 5/12cases). Distant metastatic presentation is the most unfavourable prognostic factor in ES[4]. Four patients with disseminated disease at diagnosis had complex variant translocations.

With regard to additional numerical abnormalities in ES, trisomy 8 and 12 were identified as the most frequent abnormalities [Fig. 3 and Table 2]. This is in concurrence with the previous literature findings[9,17,18]. Frequency and trend of numerical abnormalities did not differ significantly between classical and non-classical groups. Among secondary structural abnormalities, chromosomes 1 and 16 were frequently involved. Polyploidy predicts progressive disease[1], which was more frequently observed in non-classical group than in classical group. Five patients had disseminated disease at diagnosis and one with localized disease at presentation developed distant metastasis and died in three months.

Although we have successfully discovered may variant translocations and secondary chromosomal changes, specific gene partners could not be identified due to technical limitations of cytogenetics. Diagnosis of ES can be improvised by application of RT-PCR (Reverse Transcriptase Polymerase Chain Reaction), which is able to detect fusion transcripts even in small tumor samples. Gene expression arrays have discovered novel genetic markers that can potentially detect subclinical disease in histologically negative metastatic ES and thereby help in risk assessment[25].

Conclusion

The current study highlights the importance of conventional cytogenetics in the diagnosis and prognosis of ES. The proposed cytogenetic classification model may serve as a template for investigation of ES for diagnosticians and researchers, especially in centres with limited resources for molecular tests. The major inferences from the current study are the following: i) The frequency of variant translocations noted was 12% ii) Presence of variant translocation did not favour extra-skeletal origin iii) Significant correlation was noted between variant translocations/additional abnormalities and metastatic presentation iv) Chromosome 12 emerged as the second most legitimate partner with 12q24 being the most common breakpoint v) New translocation partners have been discovered, which require genetic exploration for better understanding the ES pathogenesis and to define therapeutic molecular targets.

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